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L4	L2 and (CD163)	0	L4
L3	(MAC2) adj ((158) or (48))	1	L3
L2	Morganelli.in. or Guyre.in. or goulding.in.	63	L2
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(FILE 'HOME' ENTERED AT 11:38:48 ON 14 FEB 2002)

FILE 'BIOSIS, MEDLINE, CAPLUS, USPATFULL, EMBASE' ENTERED AT 11:39:03 ON 14 FEB 2002

L1 2 S (MAC2) (W) ((158) OR (48))
L2 2 DUP REM L1 (0 DUPLICATES REMOVED)

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FILE 'BIOSIS, MEDLINE, CAPLUS, USPATFULL, EMBASE' ENTERED AT 11:45:08 ON 14 FEB 2002

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L1          166 S MORGANELLI?/AU
L2          488 S GUYRE?/AU
L3         2423 S GOULDING?/AU
L4         2989 S L1 OR L2 OR L3
L5           20 S L4 AND ((CD163) OR ((MAC2) (W) ((158) OR (48))))
L6           11 DUP REM L5 (9 DUPLICATES REMOVED)
L7           14 S (CD163) AND (CLONING OR CLONED OR CLONE?)
L8            6 DUP REM L7 (8 DUPLICATES REMOVED)

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Guyre, P. M. (1); FASEB Journal, (April 20, 2000) Vol. 14, No. 6, pp. A1143.
Meeting Info.: Joint Annual Meeting of the American Association of Immunologists and the Clinical Immunology Society Seattle, Washington, USA May 12-16, 2000

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Schaer, et al. Immunogenetics, (March, 2001) Vol. 53, No. 2, pp. 170-177.

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itiated cytoskeletal changes and cell spreading

Maria D. Mcknight and Pauline Johnson. Department
ology, The University of British Columbia, Vancouver.

cin tyrosine phosphatase essential for antigen-receptor
ation of p56lck, a tyrosine kinase in T cells. CD44 is a
ion molecule involved in a variety of biological processes
ation, lymphocyte homing, wound healing and tumor
associate with lck in T cells. It was noticed that CD45-,
T lymphoma cells could undergo morphologic changes
ie cells were immobilized by anti-CD44 monoclonal an-
change of immobilized CD45- T cells was accompanied
phosphorylation of cellular proteins. Although lck as-
h CD45+ and CD45- T cells, more lck was found to be
D45- cells. The treatment of CD45- T cells with PP2,
inhibitor, significantly reduced the CD44-antibody in-
ation as well as the morphologic change. This suggests
influence CD44-antibody triggered cell spreading. CD44
duced the tyrosine phosphorylation of cellular proteins
ay therefore regulate tyrosine phosphorylation induced
gulating the association of p56lck with CD44. This work
in the negative regulation of cytoskeletal reorganization
lleton.

ve Transcriptional Control Elements of the

and J. H. Weis. Department of Pathology, University of
Salt Lake City UT 84132

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controls sites required for Pactolus expression utilizing an
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site: this fragment was dissected into three overlapping
each. A number of suggestive factor binding sites were
GATA family members, NF-kB members, PU.1 site, etc.
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Extracts from Pactolus positive CTMC and bone marrow
A banding pattern for all the promoter fragments tested.
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S AND LPS SYNERGISTICALLY INCREASE
D163 ON CULTURED HUMAN MONOCYTES

lan. [2]P.M. Morganelli, [1]K. Wardwell, [1]V.C. Guyre,
nt of [1]Physiology and [2]Microbiology, Dartmouth Medical
756

monocyte/macrophage-specific glycoprotein with unknown
on greater than or equal to 99% of human CD14-positive
xence homology with the scavenger receptor cysteine rich
ious studies in our laboratory have shown that glucocorti-
expression on cultured peripheral blood monocytes. Using
hat recognize CD163 in flow cytometric analysis of cultured
/163, we show that bacterial lipopolysaccharide (LPS) causes
ce CD163, with a return to pre-LPS-treated levels at 24
treatment combining glucocorticoid steroid hormones and
higher expression of CD163 at 40-48 hours, with an ED50
the synthetic glucocorticoid dexamethasone and the nat-
ne cortisol synergize with LPS for up-regulation of CD163,
as that are consistent with their known relative affinities for
tor. Other steroid hormones (estradiol, progesterone, and
ect. Therefore, we conclude that synergistic up-regulation of
with LPS is a new example of a glucocorticoid-specific effect
function.

148.22

Preferential Expression of the Beta-Integrin-Like Protein Pactolus in Neutrophils: Evidence of Lymphocyte Toxicity and Strain Specificity

S.P.Garrison, A.Hojgaard, K.S.J. Elenitoba-Johnson, Y.Chen, J.J.Wels and J.H.Weis. Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84132

The Pactolus gene encodes a protein with a high degree of extracellular homology to the beta integrins. The Pactolus gene encodes three primary transcripts, two of which encode unstable truncated proteins (Pac B and C) and the third which expresses the full length, membrane bound form (Pac A). Unlike integrin subunits, the Pactolus protein lacks a functional MIDAS domain suggesting Pactolus may not require a heterodimer alpha chain for cell surface expression. Pactolus expression constructs transfected into CHO cells confer stable cell surface expression (half life >12 hrs) without any detectable endogenous partner. Transfection of the same Pactolus construct into T and B cell lines does not result in the stable expression of Pactolus suggesting that these cell types are not permissive for the expression of the protein. Pactolus expression coupled with Geneticin selection is toxic to the lymphocyte. Use of polyclonal antibodies against Pactolus has determined that the primary cell type in the mouse that expresses the gene product is the neutrophil, again without a detectable alpha chain partner. Analysis of different strains of mice has demonstrated that the level of cell surface Pactolus is controlled by strain specific alternative splicing of the Pactolus gene (A, B and C transcripts). Thus while bone marrow neutrophils from BALB/c, C57Bl6 and C3H animals all produce the same quantity of Pactolus transcripts, the C57Bl6 cells produce about 80% more of the full length form than the other strains.

148.24

HUMAN MONOCYTES EXPRESS CD163, WHICH IS UPREGULATED BY IL-10 AND IDENTICAL TO p155.

[1]T. H. Sulahian, [2]P. Högger, [1]K. Wardwell, [3]N. J. Goulding, [2]C. Sorg, [2]A. Droste, [2]M. Stahlhofen, [1]K. A. Hintz, [1]K. H. Ely and [1]F. M. Guyre. [1]Department of Physiology, Dartmouth Medical School, Lebanon, NH 03756 [2]Institute of Experimental Dermatology, Westfälische Wilhelms-University, Münster, Germany [3]Department of Biochemical Pharmacology, St Bartholomew's & Royal London School of Medicine & Dentistry, Charterhouse Square, London EC1M 6BQ

CD163 is a glucocorticoid inducible member of the scavenger receptor cysteine rich family of proteins. Previous reports have indicated that CD163 is highly expressed on human macrophages, but found on less than 50% of peripheral blood monocytes. We now show that greater than or equal to 99% of all CD14 positive monocytes express CD163, and that IL-10, like glucocorticoids, induces higher CD163 expression on cultured human monocytes. In contrast, TGF-β decreases CD163 expression. Glucocorticoid induced CD163 expression was not inhibited by anti-IL-10 and was additive with IL-10 treatment, suggesting that glucocorticoids increase CD163 expression by an IL-10 independent mechanism. In addition, we show that p155 (a previously identified monocyte/macrophage marker of unknown function) shares identity with CD163. Western blots and flow cytometric analysis of HEK 293 cells transfected with the cDNA for CD163 were positive when probed with either mAb RM3/1 (which recognizes CD163) or Mac 2-48 (which defines p155).

148.26

INCREASED PLASMA CD163 FOLLOWING CARDIAC SURGERY

[1]P.M. Guyre, [1]T.H. Sulahian, [1]P.M. Morganelli, [1]K. Wardwell, [1]K.A. Hintz, [1]A. Rassias, [1]M. Fillinger, [1]J. Sanders, [2]P. Högger, [3]N. Goulding. [1]M. Yeager. [1]Dartmouth Medical School, Lebanon, NH 03766; [2]Inst. of Experimental Dermatology, Westfallische Wilhelms-University, Munster, Germany; [3]St Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ

CD163 is a glycoprotein of unknown function expressed on circulating human monocytes and tissue macrophages. We have previously shown expression of CD163 (then known as p155) to be increased 2 to 3-fold by culture of monocytes with glucocorticoids, but not other steroid hormones (J. Immunol. 140:2298. 1988). More recently, we have found CD163 to be inducible by the anti-inflammatory cytokine IL-10, to be shed rapidly in response to bacterial lipopolysaccharide (LPS), and to be synergistically upregulated by culture of monocytes for 24-48 h with combined glucocorticoids plus LPS. We now report development of an ELISA assay to measure soluble CD163, using mAb Mac 2-158 as the capture antibody and biotinylated mAb RM3/1 as the detection antibody. We are using this ELISA to measure relative levels of CD163 in the plasma of 30 patients undergoing cardiac surgery (CABG) performed with normothermic cardiopulmonary bypass (CPB). In 4 of 4 samples tested to date, plasma CD163 increased approximately 2-fold at 60 min. following CPB, returning to slightly below baseline levels on post-operative day 1. Ongoing studies will determine the effect of preoperative treatment with methylprednisolone (15 mg/kg) on plasma CD163, and will correlate CD163 levels with plasma IL-10 and IL-6. These studies should help us gain more insight into the physiological regulation and function of CD163 *in vivo*.

148.21

CD45 regulates CD44-initiated cytoskeletal changes and cell spreading in T cells

Ruihong Li, Mojgan Jalali, Maria D. Mcknight and Pauline Johnson. Department of Microbiology and Immunology, The University of British Columbia, Vancouver, B.C., Canada

CD45 is a leukocyte protein tyrosine phosphatase essential for antigen-receptor signaling and dephosphorylation of p56lck, a tyrosine kinase in T cells. CD44 is a broadly expressed cell adhesion molecule involved in a variety of biological processes such as leukocyte extravasation, lymphocyte homing, wound healing and tumor metastasis. CD44 can also associate with lck in T cells. It was noticed that CD45-, but not CD45+, BW5147 T lymphoma cells could undergo morphologic changes and cell spreading when the cells were immobilized by anti-CD44 monoclonal antibody. The morphologic change of immobilized CD45- T cells was accompanied by an induction of tyrosine phosphorylation of cellular proteins. Although lck associated with CD44 in both CD45+ and CD45- T cells, more lck was found to be associated with CD44 in CD45- cells. The treatment of CD45- T cells with PP2, a specific Src family kinase inhibitor, significantly reduced the CD44-antibody induced tyrosine phosphorylation as well as the morphologic change. This suggests that CD45 can negatively influence CD44-antibody triggered cell spreading. CD44 mediated cell spreading induced the tyrosine phosphorylation of cellular proteins and required lck. CD45 may therefore regulate tyrosine phosphorylation induced cytoskeletal changes by regulating the association of p56lck with CD44. This work supports a role for CD45 in the negative regulation of cytoskeletal reorganization accompanying leukocyte adhesion.

148.22

Preferential Expression of the Beta-Integrin-Like Protein Pactolus in Neutrophils: Evidence of Lymphocyte Toxicity and Strain Specificity

S.P.Garrison, A.Hojgaard, K.S.J. Elenitoba-Johnson, Y.Chen, J.J.Wels and J.H.Wels. Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84132

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148.23

Identification of Putative Transcriptional Control Elements of the Murine Pactolus Gene

R.M. Maggenti, J. J. Wels, and J. H. Wels. Department of Pathology, University of Utah School of Medicine, Salt Lake City UT 84132

The murine Pactolus gene's genetic structure and sequence is similar to that of the beta integrin subunits. We initially identified Pactolus as the product of mast cells derived from bone marrow cells grown in SCF (connective tissue mast cells - CTMC) and absent in mast cells derived in IL-3 (mucosal mast cells - MMC). Further analysis has shown that the primary cells within the bone marrow of the animal that produce Pactolus are maturing and mature neutrophils. In order to elucidate tissue specific transcriptional control of Pactolus expression and to further determine the cell types where Pactolus is expressed, we analyzed the Pactolus promoter for transcriptional controls sites required for Pactolus expression utilizing an EMSA protocol. The promoter sequence included the 750 bp immediately upstream of the transcription start site; this fragment was dissected into three overlapping fragments of about 270bp each. A number of suggestive factor binding sites were noted including those for GATA family members, NF-kB members, PU.1 site, etc. Using nuclear extracts isolated from murine bone marrow cells, five distinct banding patterns were identified. Extracts from Pactolus positive CTMC and bone marrow cells have the same EMSA banding pattern for all the promoter fragments tested. Extracts from Pactolus negative MMC differ in their banding pattern at one or more sites closest to the transcription start site (-1 to -270) suggesting a possible loss of one or more transcription factors required for Pactolus transcription.

148.24

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CD163 is a glucocorticoid inducible member of the scavenger receptor cysteine rich family of proteins. Previous reports have indicated that CD163 is highly expressed on human macrophages, but found on less than 50% of peripheral blood monocytes. We now show that greater than or equal to 99% of all CD14 positive monocytes express CD163, and that IL-10, like glucocorticoids, induces higher CD163 expression on cultured human monocytes. In contrast, TGF-β decreases CD163 expression. Glucocorticoid induced CD163 expression was not inhibited by anti-IL-10 and was additive with IL-10 treatment, suggesting that glucocorticoids increase CD163 expression by an IL-10 independent mechanism. In addition, we show that p155 (a previously identified monocyte/macrophage marker of unknown function) shares identity with CD163. Western blots and flow cytometric analysis of HEK 293 cells transfected with the cDNA for CD163 were positive when probed with either mAb RM3/1 (which recognizes CD163) or Mac 2-48 (which defines p155).

148.25

GLUCOCORTICOIDS AND LPS SYNERGISTICALLY INCREASE EXPRESSION OF CD163 ON CULTURED HUMAN MONOCYTES

[1]K.A. Hintz, [1]T.H. Sulahian, [2]P.M. Morganelli, [1]K. Wardwell, [1]V.C. Guyre, [1]P.M. Guyre. Department of [1]Physiology and [2]Microbiology, Dartmouth Medical School, Lebanon, NH 03756

CD163 is a 155 kDa monocyte/macrophage-specific glycoprotein with unknown function. It is expressed on greater than or equal to 99% of human CD14-positive monocytes and has sequence homology with the scavenger receptor cysteine rich family of proteins. Previous studies in our laboratory have shown that glucocorticoids upregulate CD163 expression on cultured peripheral blood monocytes. Using monoclonal antibodies that recognize CD163 in flow cytometric analysis of cultured peripheral blood monocytes, we show that bacterial lipopolysaccharide (LPS) causes rapid shedding of surface CD163, with a return to pre-LPS-treated levels at 24 and 44 hours. However, treatment combining glucocorticoid steroid hormones and LPS causes twelve-fold higher expression of CD163 at 40-48 hours, with an ED50 >0.1 ng/mL LPS. Both the synthetic glucocorticoid dexamethasone and the naturally occurring hormone cortisol synergize with LPS for up-regulation of CD163, with dose response curves that are consistent with their known relative affinities for the glucocorticoid receptor. Other steroid hormones (estradiol, progesterone, and testosterone) had no effect. Therefore, we conclude that synergistic up-regulation of CD163 in combination with LPS is a new example of a glucocorticoid-specific effect on human immune cell function.

148.26

INCREASED PLASMA CD163 FOLLOWING CARDIAC SURGERY

[1]P.M. Guyre, [1]T.H. Sulahian, [1]P.M. Morganelli, [1]K. Wardwell, [1]K.A. Hintz, [1]A. Rassias, [1]M. Fillinger, [1]J. Sanders, [2]P. Högger, [3]N. Goulding. [1]M. Yeager. [1]Dartmouth Medical School, Lebanon, NH 03756; [2]Inst. of Experimental Dermatology, Westfalische Wilhelms-University, Munster, Germany; [3]St Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ

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Identification of the Integral Membrane Protein RM3/1 on Human Monocytes as a Glucocorticoid-Inducible Member of the Scavenger Receptor Cysteine-Rich Family (CD163)[1]

Petra Högger,[2*†] Jens Dreier,[*] Anne Droste,[*] Friedrich Buck,[‡] and Clemens Sorg[*]

The RM3/1 Ag is a membrane glycoprotein restricted to human monocytes and macrophages that evolve in the late phase of inflammation. Peptide sequence analysis of the RM3/1 protein revealed similarity to CD163, a member of the scavenger receptor cysteine-rich family. Using specific Abs (RM3/1, Ki-M8), we demonstrate an identical cellular regulation for the RM3/1 and the CD163 protein. Most notably, we show for the first time that CD163 is significantly up-regulated by glucocorticoids. In contrast, the protein is down-regulated by the immunosuppressant cyclosporin A and by phorbol esters, while the inflammatory mediator LPS has no significant influence on the expression. We describe the first isolation of a full-length cDNA of CD163 and expression of the corresponding protein. Several splice variants of CD163 exist, and we elucidated the kinetics of induction of three major mRNA splice variants by fluticasone propionate; another splice variant was proved to be unresponsive to this glucocorticoid. Taken together with a previous result showing an involvement of RM3/1 in adhesion of monocytes to the activated endothelium, we discuss that CD163 might play an important role in inflammatory processes. *The Journal of Immunology,* **1998, 161: 1883–1890.**

The RM3/1 mAb was developed in search of specific differentiation markers for mononuclear phagocytes (1). The surface Ag detected by this Ab was found to be expressed exclusively on human monocytes and macrophages, predominantly in the late phase of inflammatory processes, e.g., in experimental gingivitis (2) and in allergic contact dermatitis (1). Elucidation of the tissue distribution of the RM3/1 Ag revealed a high abundance in the human term placenta (3). It is present in skin histiocytes, livers' Kupffer cells, spleen macrophages of the red pulp, and some thymus macrophages. In addition, it is found regularly in acute and chronic inflammatory lesions (1).

The Ag is present on the surface of 15 to 30% of freshly isolated monocytes of healthy donors. Both the Ag density and the number of positive cells can be increased significantly in vitro within 48 h of incubation with dexamethasone. In contrast, inflammatory mediators such as LPS or TPA[3] were not able to enhance RM3/1 expression (4, 5). Up-regulation of the RM3/1 Ag was also demonstrated in vivo: injection of corticosteroids into primates (6) or human volunteers (4) resulted in an increase of RM3/1-positive blood monocytes up to 80% within 6 h. Induction of the RM3/1 protein was shown to be mediated by glucocorticoid receptors since a glucocorticoid antagonist inhibited up-regulation (5).

Biochemical characterization of the RM3/1 Ag revealed a 130/150-kDa glycoprotein under nonreducing and reducing SDS-PAGE conditions, respectively. The carbohydrates were *N*-linked and the glycoportion contributed about 25 kDa to the apparent molecular mass (5).

During inflammatory processes, migration of blood mononuclear leukocytes into tissues is an essential step. The recruitment of monocytes requires specific cell adhesion molecules (for review, see Ref. 7). The adhesion of different human monocyte subsets to vascular surfaces has been described for different monocyte subsets (8). RM3/1-positive monocytes exhibited pronounced adhesion to endothelial cells stimulated by IL-6 (8). Blocking experiments with the RM3/1 Ab suggested that the RM3/1 Ag, together with CD14, is involved in the adhesion of monocytes to activated endothelial cells (9).

Along with these findings, monocytes expressing the RM3/1 Ag were reported to produce an antiinflammatory factor (10, 11). This observation is coherent with earlier suggestions that the RM3/1-positive monocytes might be associated with the process of down-regulation of inflammation.

Due to the restricted availability of human monocytes, isolation and partial sequencing of the RM3/1 Ag were not feasible to date. Therefore, we developed an improved isolation procedure based on the addition of divalent cations to the solubilization mixture. The data reported in this work identify the RM3/1 protein as a member of the scavenger receptor cysteine-rich (SRCR) superfamily. Although several members of this ancient and highly conserved family have been described, possible functions of most of these proteins remain speculative (for review, see Ref. 12). We now report that the protein designated as CD163 (13) is identical with the glucocorticoid-inducible RM3/1 Ag. This is the first study showing regulation of a SRCR family member type B by various stimulants. In addition, we demonstrate the kinetics of the mRNA induction by the glucocorticoid fluticasone propionate of four major splice variants of the CD163 mRNA (14). We also describe the expression of the rCD163 protein after transfection of the predominant full-length clone into a mammalian cell line.

Institutes of *Experimental Dermatology and †Pharmaceutical Chemistry, Westfälische Wilhelms-University Münster, Germany; and ‡Institute of Cell Biochemistry and Clinical Neurobiology, University of Hamburg, Hamburg, Germany

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[3] Abbreviations used in this paper: TPA, 12-*O*-tetradodecanoyl-phorbol-13-acetate; BCIP, 5-bromo-4-chloro-indoyl-phosphate; CHO, Chinese hamster ovary; DIG, digoxigenin; NBT, nitroblue-tetrazolium-chloride; SRCR, scavenger receptor cysteine-rich; TFA, trifluoracetic acid.

0022-1767/98/$02.00

Materials and Methods

Isolation of blood monocytes

Monocytes were isolated from pooled buffy coats (Blutbank, Münster, Germany) by Ficoll-Paque (Pharmacia, Freiburg, Germany) and subsequent Percoll (Pharmacia) density-gradient centrifugation. The monocytes purity was >90%, as quantified by FACScan analysis. Monocytes were cultured at a density of 2×10^6 cells/ml in hydrophobic Teflon bags (Heraeus, Hanau, Germany) in McCoy's 5a medium (Biochrom, Berlin, Germany) supplemented with 15% FCS.

Stimulation of monocytes

Monocytes for FACScan analysis were cultivated for 16 h in presence of 10^{-7} M dexamethasone (Sigma, Deisenhofen, Germany) or 10^{-8} M of the more potent fluticasone-17-propionate (a generous gift from Glaxo Wellcome, Greenford, U.K.) (5).

Stimulation with cyclosporin A (Sigma) was performed for 16 h at a concentration of 0.1 μg/ml; stimulation with the phorbol ester TPA (Sigma) was performed for 1 h at a concentration of 10 nM.

Isolation of the RM3/1 Ag

Cells were lysed and solubilized in a one-step procedure. Monocytes were incubated with 1 to 10 mM Pefabloc SC (Boehringer Mannheim, Mannheim, Germany), 1 mM $CaCl_2$, 1 mM $MnCl_2$, and octylthioglucopyranoside (Sigma) at a protein-detergent ratio of 0.2 for 30 min at room temperature under gentle rotation. After centrifugation, the supernatant was collected. The protein extract was separated by preparative SDS-PAGE under reducing conditions, according to Laemmli (15), using an 8% running gel. Gels were stained with 0.1% Coomassie brillant blue R250 (Sigma), and slices containing the RM3/1 Ag (5) were excised and prepared for sequencing.

Protein sequencing

In-gel digestion was conducted according to the procedure described by Eckerskorn and Lottspeich (16), with the following modifications: the gel pieces were not lyophilized before digestion, but incubated in reaction buffer (25 mM Tris-HCl, pH 8.5, 1 mM EDTA) for 30 min at 37°C. Digestion was achieved overnight at 37°C with 1 μg of proteinase LysC (sequencing grade; Boehringer Mannheim) in reaction buffer. The reaction was stopped by the addition of 1 μl trifluoracetic acid (TFA), the supernatant was collected, and the gel pieces were subsequently incubated for 1 h with 100 μl of reaction buffer, TFA/acetonitrile (50:50), and acetonitrile. The supernatants were combined, concentrated to a volume of about 100 μl in a vacuum concentrator, and extracted twice with an equal volume of heptane/isoamyl alcohol (4:1) to remove traces of SDS. The peptides were then separated by narrowbore HPLC (130A; PE Applied Biosystems, Weiterstadt, Germany) on a reversed phase column (Vydac C4; 300 Å pore size; 2.1 × 250 mm). Peptides were eluted by a linear gradient (2 to 80% B in 45 min; A, water/0.1% TFA; B, 70% acetonitrile/0.85% TFA; flow rate, 200 μl/min). Peptide-containing fractions detected at 210 nm were collected manually and subjected to rechromatography on a second reversed phase column (Nucleosil C8; 300 Å; 1.6 × 250 mm; gradient as above; flow rate, 130 μl/min). Protein sequences were determined by standard Edman degradation on an automatic sequencer (473A; PE Applied Biosystems).

SDS-PAGE, Western blot, and dot blot

Proteins were separated by SDS-PAGE under reducing conditions, according to Laemmli (15), using an 8% running gel. For nonreducing and nondenaturating conditions, mercaptoethanol or DTT (both Sigma) was omitted, and the SDS concentration in the sample buffer was reduced to 0.1%.

For Western blotting, the nonstained gel was transferred to Protran nitrocellulose membrane (0.45 μm; Schleicher and Schüll, Dassel, Germany) in a semidry blotting unit (Höfer/Pharmacia) applying 0.8 mA/cm² membrane. Subsequently, proteins were detected immunochemically. Nonspecific binding to the membrane was blocked by incubation with 1% skim milk powder in PBS for 1 h under gentle shaking. After washing the membranes with PBS, the primary Ab (12 μg/ml) in TBS buffer (50 mM Tris-HCl, pH 7.6, 145 mM NaCl) containing 0.1% (BSA) was added for 1 h. Subsequently, membranes were washed with TBS containing 0.05% Tween-20 (TBST) and incubated with the alkaline phosphatase-labeled secondary Ab goat anti-mouse IgG1 (75 ng/ml in TBS) for another hour. After washing of the membrane with TBST and HP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), colorimetric detection was performed by addition of 0.15 mg/ml BCIP and 0.3 mg/ml NBT in HP buffer.

Dot blots were conducted according to the Western blot protocol, but proteins were applied directly onto the nitrocellulose membrane without preceding gel electrophoresis. Densitometric analysis of dot intensities was performed after scanning of the membrane using the National Institutes of Health ImageQuant software.

Antibodies

The mAb RM3/1 was previously generated and characterized by our group (1). The Ki-M8 Ab, mouse anti-human phagocytic macrophage II, was purchased from Bachem Biochemica (Heidelberg, Germany). The mouse isotype control IgG1 and the FITC-, Texas Red-, and alkaline phosphatase-labeled secondary Abs (goat anti-mouse IgG1) were purchased from Dianova (Hamburg, Germany).

FACScan analysis

For indirect immunofluorescence analysis, monocytes were washed with cold PBS, and incubated with 1% BSA for 30 min at 4°C. Then cells were incubated with the primary Ab (3 μg/ml) for 45 min at 4°C. Mouse IgG1 was included as isotype control at the same concentration. Subsequently, monocytes were washed with PBS and incubated with FITC-labeled secondary Ab goat anti-mouse IgG1 in 1% BSA for 30 min at 4°C. Propidium iodide (1 mM in PBS) was added for the last 2 min of incubation to determine cell viability and exclusion of dead cells. The fluorescence intensity of 10^4 vital cells was measured by FACScan analysis (Becton Dickinson, Heidelberg, Germany). The parameters used were 488 nm excitation wavelength, 250 mW, and logarithmic amplification. The Ag density and the number of RM3/1-positive cells corrected for isotype control were obtained from the main fluorescence channel at 510 to 530 nm using Lysis software (Becton Dickinson).

Cell culture and transfection of CHO cells

CHO DUKX B1 cells (ATCC CRL 9010) were maintained in alpha medium with desoxy- and ribodesoxynucleosides (Life Technologies, Gaithersburg, MD) complemented with 10% FCS (PAA Laboratories, Linz, Austria) and 2 mM glutamine (Biochrom). Cells were transfected using the cationic lipid reagent Dotap (Boehringer Mannheim). Transfection was performed as described in the product protocol. Briefly, 5×10^5 cells were seeded onto 60-mm dishes 1 day before transfection. Immediately before transfection, cells were rinsed with medium without FCS. For each 60-mm dish, 5 μg of plasmid DNA (clone CD163A-6) and 30 μl Dotap were diluted to 50 μl and 100 μl, respectively. The diluted DNA and Dotap were mixed gently and incubated for 15 min at room temperature. The DNA/Dotap mixture was then diluted with 3 ml medium without serum and added to the cells. After incubation at 37°C in humidified CO_2 incubator for 2 h, 3 ml of Ham's F12 medium with 20% FCS was added. The cells were fixed for immunofluorescence after further incubation at 37°C for 24 h.

Immunofluorescence

For immunofluorescence studies, transfected CHO cells were plated on coverslips and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. For localization of transiently expressed rCD163, cells were incubated with 4 μg/ml Ki-M8 in blocking solution for 60 min at room temperature. This was followed by a 20-min incubation with 1:150 Texas Red-conjugated anti-mouse IgG (stock concentration 1.5 mg/ml) in blocking solution. Cells were viewed using a fluorescence microscope (Axioskop; Zeiss, Jena, Germany); the enlargement scale was 1:8750.

Northern hybridization

Total RNA was prepared from fluticasone propionate-stimulated and control monocytes, as previously described (17). A quantity amounting to 10 μg of total RNA per sample was fractionated on 1% agarose formaldehyde gels and transferred to Hybond N nylon membranes (Amersham, Arlington Heights, IL) with 20× SSC (1× SSC: 0.15 M NaCl and 15 mM sodium citrate) using a LKB 2016 VacuGene blotting apparatus. Antisense RNA probes for Northern hybridization were generated by the DIG RNA labeling kit (Boehringer Mannheim) using linearized DNA templates and T7 RNA polymerase, as described by the manufacturer. Prehybridizations were performed at 68°C for 1 h in a high SDS hybridization buffer (7% SDS, 5× SSC, 50% formamide, 50 mM sodium phosphate, pH 7, 2% casein, and 0.1% *N*-lauroylsarcosine). The heat-denatured probe (10 min at 95°C) was added to the prehybridization solution (100 ng/ml), followed by gentle agitation at 68°C for 16 h. The nylon membranes were washed twice for 10 min at room temperature in a 2× SSC, 0.1% SDS solution, and twice for 15 min at 68°C in a solution consisting of 0.1× SSC and 0.1% SDS. The hybridization results were visualized by chemiluminescent detection

Table I. *Sequences of five RM3/1 peptides after LysC digestion and their positions in the published CD163 sequence*

Determined Peptide Sequence	Published Sequence[a]	Position no.
ELRLVDGENK	KELRLVDGENK	45–54
XSYQVYSK	KTSYQVYSK	417–424
XMXIPM	KAMSIPM	878–883
A/X M/P D/X I/T P/L M/X Q/X V/X P/(D)	KGPDTLWQCP	893–901
RLAXPXE(E)	KRLASPSEE	908–915

[a] Sequence from Reference 14. Amino acids which could not be determined with certainty are marked with "X."

with anti-DIG F(ab')$_2$ fragments conjugated with alkaline phosphatase and substrate CSPD, as described by the manufacturer (Boehringer Mannheim). Equal loading of samples was controlled by hybridization of RNA with an actin antisense RNA probe.

Construction and screening of a monocyte cDNA library

Human monocytes were cultured in Teflon bags at cell densities of 2 × 10^6/ml, as previously reported (1). After 12 h of incubation, cells were stimulated with fluticasone propionate (10^{-8} M) and cultured for 1 day. Pure monocyte populations were isolated by immunomagnetic separation using Dynabeads M-450 CD14, succeeded by direct poly(A$^+$) RNA isolation with Dynabeads oligo(dT)$_{25}$ (Dynal GmbH, Hamburg, Germany), according to the instructions of the manufacturer.

Construction and ligation of cDNA into the vector Lambda Uni-ZAP Express were prepared according to the manufacturer's specifications (Stratagene, Heidelberg, Germany). The DNA was packaged by Gigapack III gold extract and amplified in *Escherichia coli* strain XL1-Blue MRF'. An amplified library with 6.5 × 10^5 independent clones and a mean insert size of 3.5 kb (0.5 to 6 kb) was obtained.

The library was spread as a monolayer on the XL1-MRF' strain with a titer of ~2.5 × 10^4 clones/138-mm plate, and the plaques were subsequently transferred to Hybond N filters (Amersham). The filters were screened with a DIG-labeled PCR fragment, generated by RT-PCR with primers CD163un 5'-CCCGTCGACAATGAGCAAACTCAGAATG GTG-3' (CD163A, positions 86 to 107) CD163ln 5'-CCCCGTCCTTG GAATTTGATCTCTATT-3' (CD163A, positions 599 to 623). Filters were washed at high stringency (0.1× SSC, 0.1% SDS, 68°C). Colorimetric detection was performed with NBT and BCIP. The resulting 29 positive phages were plaque purified, and the pBK-CMV (Stratagene) phagemid vectors containing CD163 inserts were recovered by in vivo excision, according to the manufacturer's instructions. After restriction endonuclease mapping of the cDNA clones, the nucleotide sequences were established by sequencing both strands of the DNA insert. Two full-length cDNA clones were obtained, representing the coding region of the major variant of CD163 (accession number Z22968 (14)). The clone CD163A-6 (positions 38 to 3703) was used for transfection and expression experiments.

cDNA synthesis and RT-PCR

cDNA was synthesized from 2.5 μg of total RNA with oligo(dT)$_{12-18}$ (Pharmacia) as template primer using M-MuLV reverse-transcriptase Superscript II (Life Technologies), as described by the manufacturer. The reaction was conducted in a final volume of 50 μl containing 1 μl of the transcribed cDNA probe (5%), 200 μM of each dNTP, 1× PCR buffer including 1.5 mM $MgCl_2$ (TaKaRa Biomedicals, Shiga, Japan), 0.4 μM forward and reverse primers, and 2.5 U *Taq* polymerase (TaKaRa) covered with two drops of mineral oil (M 3516; Sigma). All CD163 amplimers were amplified simultaneously with β-actin as internal standard (multiplex PCR). Forward and reverse primers for β-actin amplification were chosen from different exons to detect a possible contamination with nuclear DNA, resulting in PCR products of 222 bp (cDNA) and 430 bp (nuclear DNA), respectively. The respective primer pairs were for human β-actin (GenBank-EMBL accession number M10277): β-ACTu (5'-TTCCAGCCTTC CTTCC-3'; positions 2449 to 2464 (exon 3)) and β-ACTl (5'-TTGCGCT CAGGAGGAGCAA-3'; positions 2861 to 2879 (exon 5)), for CD163 variant E1 (accession number Z22971 (14)) CD163E1 u (5'-CCCAAGCTT CTCAAGTAAGACCCAGAAAA-3'; positions 1817 to 1836) and CD163E1l (5'-CCCGAATTCCAAGCGAATTTCTGTGTATC-3'; positions 1902 to 1921), for CD163 variant AC2 (accession number Z22970 (14)) CD163AC2u (5'-CCCAAGCTTAATCACACATGCTTTTCTTC-3'; positions 4478 to 4497) and CD163AC2l (5'-CCCGAATTCTTATA AATTCAGCAGCAGTC-3'; positions 4850 to 4869), and for CD163 variant A (accession number Z22968 (14)) and CD163 variant AC1 (accession number Z22969 (14)) CD163RTu (5'-GCATTATTCTTCTTGACTA-3'; positions 3285 to 3303) and CD163RTl (5'-CCTTGAAAGTCT CATATAC-3'; CD163A, positions 3656 to 3674, and CD163AC1, positions 3739 to 3757). Restriction sites added to the primers are underlined. PCR was assayed at two different cycle numbers by removing one-half of the reaction volume at appropriate time points during amplification. This allowed the control of saturation effects of the PCR, which made it possible to better judge quantitative differences between samples. As control, PCR reactions were performed without addition of template and on RNA samples that had been incubated in the absence of reverse transcriptase. The reactions were conducted in a RoboCycler 40 temperature cycler (Stratagene). The reactions were incubated for 3 min at 94°C, followed by 22 to 32 cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 50°C, and extension for 1 min at 72°C. An aliquot of the PCR reaction (5 μl) was loaded in parallel with the m.w. marker (pBR322, *AluI* digested; MBI Fermentas, St. Leon-Rot, Germany) on a 1.5% agarose gel containing ethidium bromide, and the gel was photographed under UV light. The relative intensity of bands was measured densitometrically, and the results were expressed as a ratio of the CD163:β-actin band intensities.

Results

Isolation and sequencing of the RM3/1 Ag

The most potent glucocorticoid, fluticasone propionate, was used to increase both the Ag density and the number of positive human monocytes in vitro (5). Monocytes were extracted with detergent-containing buffers of varying concentrations of divalent cations. Subsequently, the amount of intact RM3/1 as recognized by the mAb was determined in the supernatant. Experiments with addition of EDTA as inhibitor of metalloproteinases resulted in reduced reactivity of the RM3/1 Ag in dot blots. Systematic evaluation of the effect of EDTA, calcium, magnesium, and manganese on protein yield showed a clear dose-dependent correlation between amounts of divalent cations and improvement of RM3/1 yield (data not shown). Addition of 1 to 10 mM calcium and manganese was statistically significantly superior vs control (no additives) and vs equimolar concentrations of EDTA (ANOVA and subsequent Fisher PLSD test, $p \leq 0.05$). Thus, for subsequent experiments, 1 mM calcium chloride and 1 mM manganese chloride were added to the solubilization mixture.

After preparative SDS-PAGE (5), gel slices containing the RM3/1 protein were excised and prepared for sequencing. Following digestion with LysC and HPLC separation, five peptide sequences were obtained (Table I). Sequences revealed homology with the CD163 protein sequence. Based on 1116 amino acid residues (1076 amino acids in the processed protein) of CD163, the derived sequences were well distributed over the protein and showed either complete identity or, in cases in which the amino acids could not be determined with certainty, a high degree of similarity with the CD163 protein.

Western blots with RM3/1 and Ki-M8 Abs

Western blots with the RM3/1 Ab were not successful to date, and it appeared that the Ab detected only the nondenatured protein. The protocols for SDS-PAGE and subsequent blotting were optimized for detection of RM3/1 Ag in Western blots. The SDS-PAGE was performed under nondenaturating and nonreducing

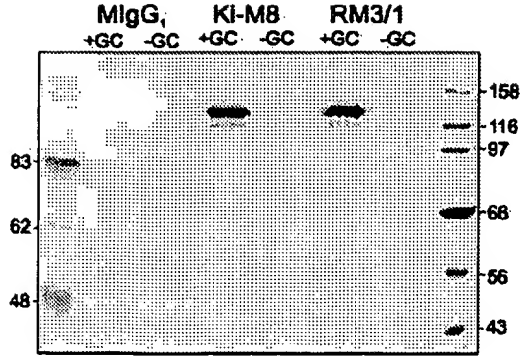

FIGURE 1. Western blot of glucocorticoid-treated and nontreated human monocytes. Glucocorticoid-induced (GC^+) and noninduced (GC^-) monocyte extracts were immunostained with Abs Ki-M8, RM3/1, and MIgG1 for nonspecific control.

conditions. Nonstimulated and glucocorticoid-stimulated monocytes were solubilized in the presence of calcium and manganese and used in Western blots (Fig. 1). The Ab Ki-M8 has been described to recognize CD163 (14). Both Abs, RM3/1 and Ki-M8, clearly detected the same protein, which has an apparent molecular mass of 130 kDa, as described before for the RM3/1 Ag under nonreducing conditions (5). Nonstimulated monocytes gave a weak signal with both the RM3/1 and the Ki-M8 Ab, whereas stimulated monocytes gave an intensive signal after immunostaining.

FACS analysis of the regulation of CD163 expression

FACScan analysis of nonstimulated monocytes (control) and stimulated monocytes revealed that all stimulants used influenced the Ag expression in the same manners. Although the extent of Ag density was not identical after immunostaining with RM3/1 and Ki-M8, there was a significant increase in the number of positive cells after addition of fluticasone propionate, whereas a clear decrease of cells carrying this Ag was observed after TPA and cyclosporin A stimulation (Fig. 2).

Screening of a monocyte cDNA library for CD163 full-length clones and expression of CD163 in CHO cells

As the full-length clone of CD163 had not been isolated before (14), we attempted to clone it. The cDNA library was constructed

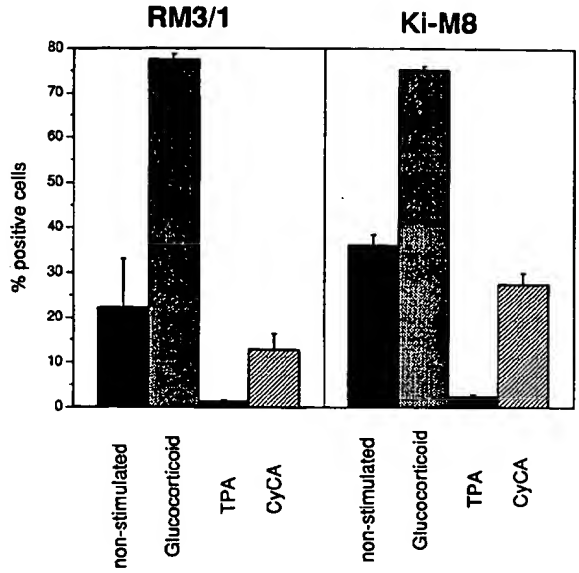

FIGURE 2. Modulation of CD163 expression. Result of a FACScan measurement of 10^4 vital human monocytes treated with the glucocorticoid fluticasone propionate (16 h, 10^{-8} M), the phorbol ester TPA (1 h, 10 nM), and cyclosporin A (CycA, 16 h, 0.1 μg/ml), and immunostaining with Abs RM3/1, Ki-M8, and MIgG1 for isotype control. Number of positive cells, corrected for isotype control, was obtained from the main fluorescence channel using Lysis software. Results indicate mean and SD of four independent measurements of a representative experiment.

in vector Lambda ZAP Express (Stratagene) made from oligo(dT)-primed cDNA of fluticasone propionate-stimulated human monocytes. The library was screened with a 0.5-kb PCR-generated DNA fragment (primer pair CD163un/CD163ln) located in the 5′ region of the CD163 sequence, as indicated in Figure 3. Positive Lambda clones were purified, and the pBK-CMV phagemid vectors containing CD163 inserts were recovered by in vivo excision. After restriction endonuclease mapping of the cDNA clones, the nucleotide sequences were determined. Of 29 isolated cDNA clones, two contained the complete nucleotide sequence, representing the coding region of the major CD163 variant (accession number

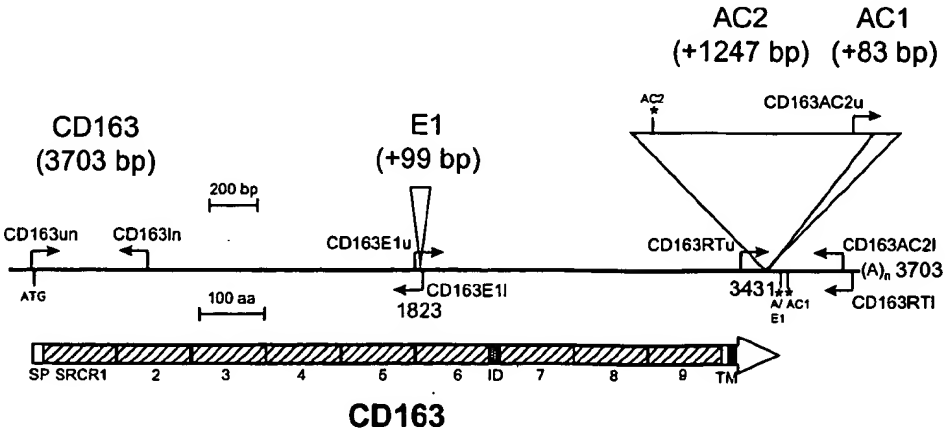

FIGURE 3. Map of CD163 cDNA and its splice variants. Insertions at position 1823 (E1, 99 bp) and position 3431 (AC2, 1247 bp; AC1, 83 bp) are marked. Start (ATG) and stop codons (*) are indicated for each splice variant. The positions of oligonucleotides used for mRNA expression studies (RT-PCR) and probe synthesis for screening the cDNA library are indicated by arrows. The region encoding the structural gene (CD163A, a major splice variant) is marked on the linear map consisting of a putative signal peptide (SP; 40 amino acids), nine scavenger receptor cysteine-rich domains (SRCR; approximately 110 amino acids), and a transmembrane segment (24 amino acids), followed by a cytoplasmic domain (49 amino acids). The interdomain spacer (ID) consisting of 31 amino acids is located between the sixth and seventh SRCR domain.

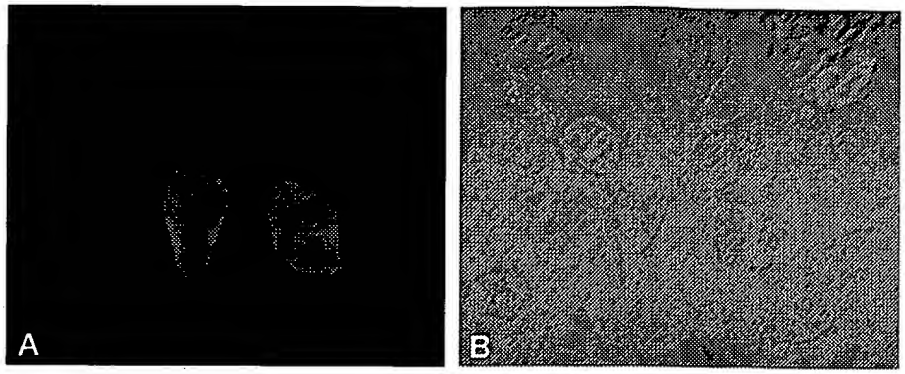

FIGURE 4. Expression of rCD163 in transiently transfected CHO cells. Transfection with CD163A-6 cDNA resulted in a positive immunostaining with Ki-M8 and RM3/1 Abs after 24 h. Cells were not permeabilized before immunostaining (*A*, Ki-M8 Ab) to demonstrate Ag expression at the cells' surface. *B* is a phase contrast image of the same cells. Scale, 1:8750.

Z22968 (14)). The clone CD163A-6 (positions 38 to 3703) was used for transfection and expression experiments. The intactness of the sequences was confirmed by nucleotide sequencing and expression experiments in CHO cells. Transient transfection of CHO cells with CD163A-6 resulted in positive immunostaining of cells with Ki-M8 Ab (Fig. 4*A*). A control experiment with nonpermeabilized cells confirmed that the Ag was expressed at the cells' surface (Fig. 4*B*). Cells immunostained with RM3/1 Ab were of identical appearance as cells stained with Ki-M8. There was no signal observed after immunostaining with isotype control mouse IgG1 and with nontransfected cells (data not shown).

Glucocorticoids induce CD163 mRNA in monocytes

Northern blot analysis revealed CD163 transcripts of about 3.7 kb for splice variant CD163-A, and 3.8 kb for splice variant CD163-AC1. Both transcripts appear as a single band (Fig. 5). Transcripts' sizes are in agreement with the predicted size of the cDNA (14). Stimulation of peripheral blood monocytes with the glucocorticoid fluticasone propionate leads to a significant increase of mRNA expression encoding CD163. Hybridization with variant specific probes revealed identical time courses for induction of splice variants A and AC1 (data not shown). Time-course experiments detected maximal induction of CD163 gene transcription after 8 h. Then the level markedly decreased toward 24 h. Analysis of additional time points (10, 12, 16, and 36 h) indicated a decrease of expression 8 h after induction (data not shown). Weak expression was detected at 0 h, with a slight increase during incubation of the cells without glucocorticoid stimulation.

CD163 splice variant expression

RT-PCR was used for analysis of the expression of CD163 splice variants at the mRNA level. Several splice variants were described and detected in an LPS-stimulated human monocyte library (14). The insertion at position 3431 of 1247 bp (AC2) and 83 bp (AC1), respectively, results in protein variants with alternative cytoplasmic domains as compared with the major form (A). In addition, the surface Ag variant E1 exhibits a 99-bp insertion at position 1823 (Fig. 3).

Our aim was to analyze the expression and relative abundance of the major form (A) and its splice variants (AC1, AC2, E1) in glucocorticoid-stimulated and control monocytes. As outlined in the experimental procedures, all of our experiments were standardized by β-actin coamplification in multiplex PCR (222 bp). Splice variant-specific sequences were amplified by RT-PCR using primers based on the cDNA sequences deposited in the GenBank/EMBL database (Fig. 3). Using the primer pair CD163RTu/CD163RTl, PCR products with expected size of 390 bp (A, E1), 454 bp (AC1), and 1718 bp (AC2) should be amplified. Because PCR conditions were chosen for amplification of DNA fragments below 500 bp, no AC2 product was detected with these primers. As shown in Figure 6*A*, two CD163-specific products of predicted size were amplified. For AC2 detection, primer pair CD163AC2 u/CD163AC2l was used, amplifying a 392-bp fragment (Fig. 6*B*). To quantify E1 transcripts, primers CD163E1 u and CD163E1l were used, generating a 124-bp PCR product. After 32 cycles, the E1 PCR product was detected in significant amounts, whereas 22 cycles amplified the E1 product at comparatively low levels (Fig. 6*C*).

As shown in Figure 6, glucocorticoid stimulation of monocytes resulted in an up-regulation of mRNA expression of variants A, AC1, and AC2. Maximal induction was observed after 8 h, and expression declined by 24 h. As already observed for Northern blot analysis, later time points (10, 12, and 16 h) revealed a decrease of expression after 8 h (data not shown). In contrast to these results, no significant increase in the amount of mRNA was observed for splice variant E1 (Fig. 6*C*).

The relative abundance of CD163 splice variants is shown in Figure 7, with maximal mRNA expression after 8 h of glucocorticoid stimulation (A, 100%; AC1, 75.7%; AC2, 38.5%; E1, 6.8%) and subsequent decrease (24-h stimulation; A, 86.8%; AC1, 68%; AC2, 25.9%; E1, 3.7%).

Discussion

In the present study, we identified the RM3/1 Ag, previously only described immunologically and biochemically, as a member of the scavenger receptor cysteine-rich superfamily, recently designated as CD163 (13). Most notably, we show for the first time that CD163 is induced significantly by the antiinflammatory glucocorticoids, whereas it is down-regulated by the immunosuppressant

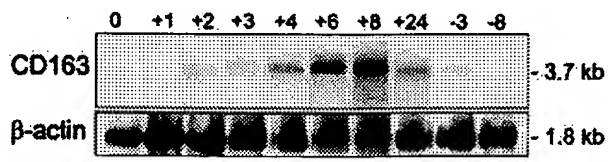

FIGURE 5. Time course of glucocorticoid-induced increase of CD163 mRNA levels. Northern blot analysis was performed with total RNA extracted from peripheral blood monocytes treated with (+) or without (−) fluticasone propionate after 1 to 24 h. Denatured RNAs were fractionated through a formaldehyde agarose gel, blotted to nylon membrane, and probed with DIG-labeled antisense RNAs. The blot was subsequently probed for β-actin transcript. The approximate size of specific transcripts was determined against an RNA molecular size ladder. What appears to be a single band at 3.7 kb are the splice variants A with 3.7 kb and AC1 with 3.8 kb.

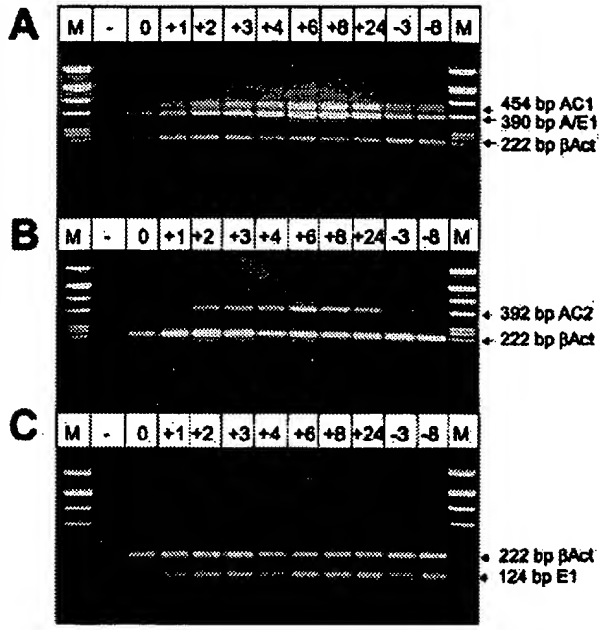

FIGURE 6. mRNA expression of CD163 splice variants after glucocorticoid stimulation analyzed by RT-PCR. Total RNA for cDNA synthesis was isolated from monocytes treated with (+) or without (−) fluticasone propionate after 1 to 24 h. Human monocytes expressing gene transcripts for CD163 variants A and AC1 (*A*), for AC2 (*B*), and for E1 (*C*). Coamplifications with specific primers for β-actin and CD163 variants were performed for 22 (*A* and *B*) and 32 cycles (*C*), respectively. As negative control (−), no template was added to the PCR reaction. Ethidium bromide strain of the amplification products. Results of one representative experiment are given. Each sample was repeated a minimum of three times, and parallel coamplifications of the same cDNA samples were performed to detect CD163 splice variants.

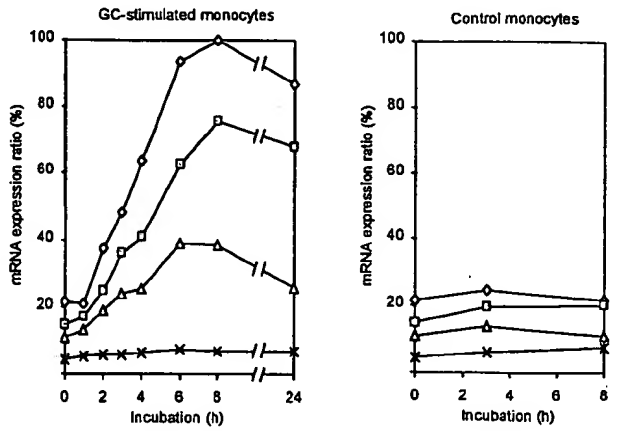

FIGURE 7. mRNA expression ratio of CD163 splice variants. Relative abundance of splice variants A (diamond), AC1 (square), AC2 (triangle), and E1 (cross) was estimated as a ratio of the CD163:β-actin band intensities at 22 PCR cycles, as determined by densitometry. Results are presented as percentage of the normalized intensity of the RT-PCR band with maximal intensity (CD163A, +8 h). Results of one representative experiment are given.

cyclosporin A. The surface expression is clearly decreased by the inflammatory mediator TPA, whereas LPS has almost no influence on the expression (5). In addition, we can now assign an involvement in adhesion processes of monocytes to the activated endothelium (9) to CD163, a member of the SRCR superfamily.

The discovery of a significant increase in recovery of the Ag by addition of divalent cations, preferentially calcium and manganese, to the solubilization mixture initiated the successful isolation. Thus, calcium or manganese is required for structural integrity, enhancing protein stability during isolation and/or preserving the epitope recognized by an Ab. A protection from proteolysis and enhancement of structural stability by divalent cations have been shown for some proteins (18, 19).

Sequencing of peptides derived from the purified RM3/1 Ag revealed sequence identity with the previously described M130 (= CD163) protein (14, 20). This Ag was only detected on cells of the monocyte/macrophage lineage. This is in complete agreement with results previously obtained for the RM3/1 Ag in normal human tissues (1). Interestingly, only few cell lines express CD163. Expression has been described for U937 cells after prolonged stimulation with phorbol esters and for SU-DHL-1 cells (20).

Several experiments were performed to confirm that the previously defined RM3/1 Ag is identical with the CD163 protein. A Western blot of solubilized nonstimulated and glucocorticoid-stimulated human monocytes resulted in superimposable bands originating from staining with RM3/1 and Ki-M8 Abs, respectively. In addition, immunostaining and subsequent FACScan analysis revealed corresponding results for both Abs. Regulation of protein surface expression by the glucocorticoid fluticasone propionate, the phorbol ester TPA, and the immunosuppressant cyclosporin A was equivalent for the protein recognized by RM3/1 and Ki-M8, respectively.

For further molecular characterization of the CD163 gene, we isolated the cDNA from a fluticasone propionate-stimulated monocyte library and transfected CHO cells with the predominant clone variant CD163A. Law et al. (14) isolated only partial cDNA fragments of CD163. Thus, our study is the first description of cloning and expression of a full-length CD163 clone.

The genomic localization and composition of the exon-intron structure of the CD163 gene are not known yet, but the existence of different splice variants for CD163 has been reported previously (14). Alternative splicing is widespread, it is described for several human surface receptors, and it appears to be a common property among proteins with scavenger receptor domains, e.g., for the scavenger receptor class B type 1 (SR-B1), a sponge scavenger receptor, and CD6 (21–23). Alternative splicing of the same transcript resulted in different cytoplasmatic domains (21, 23) that could potentially modify signal transduction. Evaluation of molecular recognition motifs for protein kinases (24) in the cytoplasmatic domain of CD163 revealed that, for example, the variants A and E1 of CD163 have one potential substrate site for protein kinase C, and the variants AC1 and AC2 each have two protein kinase C phosphorylation sites. Further studies are in progress to determine the functional role of CD163 isoforms.

Using RT-PCR, we analyzed kinetics of induction and relative abundance of four CD163 splice variants. After stimulation with fluticasone propionate, mRNA expression increased rapidly for variant A, AC1, and AC2, peaking after 8 h. Similar expression kinetics have been described for other glucocorticoid-induced gene transcripts (25, 26). The expression of the variant E1, however, appears to be constitutive at low levels and is not inducible by glucocorticoids. No time-specific differences in the relative abundance of CD163 splice variants were apparent. In human monocytes, variant A represented the major mRNA species with 44% of total CD163 mRNA, whereas variants AC1 and AC2 represented 31 and 20%, respectively. The minor mRNA species, denoted E1, was detected at low levels (5% of total CD163 mRNA). In Nothern blots, splice variants A and AC1 were detected. Time course of

induction is in complete agreement with data derived from RT-PCR experiments. Since the alternative transcripts A, AC1, and AC2 were detected at significant levels, it may be presumed that the variant proteins derived from these transcripts are also produced. However, this could not be proven yet.

Based on the spacing of the cysteine residues in the CD163 molecule, it has been assigned to the group B of the SRCR family (12). Within this group, it shares structural homology with the WC1 Ag, CD5, CD6, and Spα (12, 27). There is not much information available about the regulation of these structurally closely related proteins. The effect of dexamethasone has been investigated in bovine lymphocyte populations that express the WC1 Ag (28). The WC1 Ag density on PBMC increased during glucocorticoid injections, while there was a loss in circulating WC1-positive lymphocytes (28). In humans, a similar increase of RM3/1 (CD163)-positive monocytes has been reported 6 h after injection of dexamethasone (4); there is, however, no information about the further time course.

As for the less closely related scavenger receptors, e.g., the SR-B1, glucocorticoids were reported to decrease the expression of these proteins (29, 30), whereas inflammatory mediators such as phorbol esters increased receptor expression in smooth muscle cells (31). In contrast, it has been documented that the expression of CD163 decreases upon stimulation with phorbol esters (5), and also with cyclosporin A (9).

The function of members of the type B SRCR family is not defined conclusively and fully understood yet. WC1 is involved in γδ T cell regulation (32–34). CD5 and CD6 modulate T cell activation; Spα is thought to regulate monocyte functions (27). These proteins obviously exert their functions after binding to a specific ligand: CD5 binds to CD72 (35, 36), CD6 to ALCAM (37, 38), a new member of the family, and gp340 binds calcium dependently to lung surfactant protein D (39). No ligand has been defined yet for CD163 and the related WC1 and Spα.

However, CD163 seems to be involved in adhesion to activated endothelium cells (8, 9). Together with CD14, it has been shown to promote adhesion via a selectin- and integrin-independent pathway, which could be blocked by addition of Abs. Therefore, CD163-positive monocytes have been discussed to promote monocyte infiltration into inflammatory tissues by a nonclassical adhesion mechanism (9). In contrast, glucocorticoids have been reported to inhibit cellular adhesion by down-regulation of several adhesion molecules and to inhibit leukocyte binding to endothelium (40).

It has been proposed early that the RM3/1 (CD163) protein might have some function in the down-regulatory phase of the inflammatory process (1). RM3/1-positive monocytes were later found to produce a novel antiinflammatory factor (11, 41). Recently, it has been reported that RM3/1 (= CD163)-positive monocytes actively inhibit proliferation of $CD4^+$ T cells and PBL (42). In conclusion, CD163 appears to play an important regulatory role in immunologic processes.

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Hintz, K. A. (1); Sulahian, T. H. FASEB Journal, (April 20, 2000) Vol. 14, No. 6, pp. A1143.
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BRIEF COMMUNICATION

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Philippe Linnscheid · Hannah Staege · Christoph Müller
Gabriele Schoedon · Andreas Schaffner**

Molecular cloning and characterization of the mouse CD163 homologue, a highly glucocorticoid-inducible member of the scavenger receptor cysteine-rich family

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Keywords Mouse · Macrophage · CD163 · Scavenger receptor · Dexamethasone

CD163 is a glycoprotein belonging to the scavenger receptor cysteine-rich superfamily (SRCR) expressed on cells of the monocyte/macrophage lineage. The protein is induced by the anti-inflammatory mediator, dexamethasone, and is proposed to be associated with the downregulatory phase of inflammatory reactions. However, the biological properties of the protein are poorly characterized. In the present report, the mouse *CD163* cDNA (*mCD163*) was cloned from dexamethasone-treated peritoneal macrophages using a reverse transcription-PCR-based screening method. The predicted polypeptide sequence of the type I transmembrane glycoprotein consists of a 38-amino acid signal peptide, nine SRCR domains, one transmembrane domain, and a short cytoplasmic tail. Sequence variance analysis of all mouse and human CD163-SRCR domains defined a spatial cluster of evolutionarily conserved residues, which could be mapped to a distinct surface area putatively involved in inter- or intramolecular interactions. Determination of *mCD163* mRNA expression by quantitative real-time PCR and RNAse protection assay revealed a more than tenfold induction by dexamethasone and three- to fivefold induction by interleukin-10. In situ hybridization of mouse tissues revealed constitutive expression of *mCD163* mRNA in cells of the monocyte/macrophage series, with the highest expression level observed in Kupffer cells of the liver.

Since the cloning of the mouse type I scavenger receptor in 1990 (Freeman et al. 1990), several cDNAs have been cloned encoding proteins that are homologous to the scavenger receptor cysteine-rich (SRCR) domain found at the C-terminus of this receptor. Members of the SRCR superfamily are cell surface or secreted proteins containing one or more domains highly homologous to the SRCR domain. The SRCR superfamily can be divided in two groups, A and B, based on the number and pattern of cysteine residues in each SRCR domain. Group A proteins include the macrophage scavenger receptor A (Freeman et al. 1990), Mac-2-binding protein (M2BP) (Koths et al. 1993), complement factor I (Goldberger et al. 1987), enterokinase (Kitamoto et al. 1995), lysil oxidase-related protein (Saito et al. 1997), the sea urchin speract receptor (Dangott et al. 1989), and MARCO (Elomaa et al. 1995). Group B includes CD5 (Jones et al. 1986), CD6 (Aruffo et al. 1991), WC1 (Wijngaard et al. 1992), Spα (Gebe et al. 1997), Pema-SPERG (Mayer and Tichy 1995), Ebnerin (Li and Snyder 1995), CRP-ductin (Cheng et al. 1996), Hensin (Takito et al. 1996), DMBT1 (deleted in malignant brain tumors) (Mollenhauer et al. 1997), gp-340 (Holmskov et al. 1999), and CD163 (Hogger et al. 1998; Law et al. 1993).

The functional properties of many members of the group B SRCRs are relatively poorly characterized.

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Several of these proteins are expressed by leukocytes and have been implicated in the development and regulation of the immune system, either by modulating effector cell activity or effector cell abundance by the inhibition of apoptosis (Miyazaki et al. 1999). Others have been proposed as candidate tumor suppressor genes implicated in the pathogenesis of brain tumors and various tumors of the lung and gastrointestinal tract. (Holmskov et al. 1999; Mollenhauer et al. 1997; Mori et al. 1999; Wu et al. 1999)..

Human CD163 is a type I cell surface protein belonging to the group B SRCR superfamily and is expressed on cells of the human monocyte/macrophage lineage (Hogger et al. 1998; Law et al. 1993). Expression of the CD163 antigen is regulated by anti-inflammatory mediators in vivo and in vitro (Buechler et al. 2000; Zwadlo-Klarwasser et al. 1990). Glucocorticoids and the anti-inflammatory cytokine interleukin (IL)-10 induce high levels of human CD163 (hCD163) expression on monocytes and macrophages, while the expression is completely abrogated by IL-4, IL-13 and the nuclear receptor PPARγ (peroxisome proliferator-activated receptor γ) agonist 12-deoxy-prostaglandin J2 (Schaer et al., unpublished data). Immunohistological studies have shown an accumulation of hCD163-positive macrophages during the healing phase of acute inflammatory reactions and in chronic inflammatory diseases such as psoriasis (Djemadji-Oudjiel et al. 1996; Zwadlo et al. 1987), suggesting a role for this glycoprotein in the downregulation of the inflammatory process. However, the underlying mechanism resulting in the observed downregulation of the inflammatory process has been poorly characterized due to the lack of a suitable animal model. Glucocorticoids are the most widely used immunosuppressive and anti-inflammatory agents in clinical medicine, yet many of their pharmacological activities involved in the modulation of the immune system are still poorly understood. In this report, we present the cloning and molecular characterization of the mouse *CD163* homologue, and propose this system as a valuable model for the improved characterization of the CD163 glycoprotein in vivo.

We chose an RT-PCR-based cloning strategy using a set of gene-specific primers randomly distributed over the *hCD163* sequence. Total cellular RNA was isolated from cultured mouse peritoneal macrophages with the QIAgen RNA Mini Kit (Qiagen) and reverse transcribed into cDNA with oligo(d)T-primers and M-MuLV Reverse Transcriptase (Stratagene first-strand synthesis kit). Amplification of cDNA from dexamethasone-treated macrophages with 1 out of 15 primer pairs by a standard PCR procedure (forward-primer: 5′-CTCACTGGGACATAGAAGATGC; reverse-primer: 5′-GCCTCTGTAATCTGCTCAGG) yielded a 220-bp fragment with high homology to exon 8 of the *hCD163* gene (mFAT 1.2). The sequence of this fragment served as a template for primer extension PCR toward both ends of the cDNA (SMART RACE kit; Clontech). Two fragments of 2 and 2.5 kb were amplified in the 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE PCR reactions, respectively (5′-RACE primer: 5′-TCCAGTGCAGT GGAACATGTGACTCCAGAC; 3′-RACE primer: 5′-TGGGGTTGCCCAATCTATTCCAGAAGGAGC; anchor primers: provided with the kit) (Fig. 1). Sequence specificity was demonstrated for both fragments by nested PCR using internal primer pairs (5′ nested primer: 5′-CTGACCAGCTCCTTTCCCAAAA TGTGCTCC; 3′ nested primer: 5′-AGGTCTGGA GTCACATGTTCCACTGCACTG). All fragments were cloned into a suitable PCR cloning vector (pCR2.1 Topo-TA; Invitrogen). Two independent clones from two different PCRs were isolated and sequenced from both termini by primer walking to determine the definite nucleotide sequence of

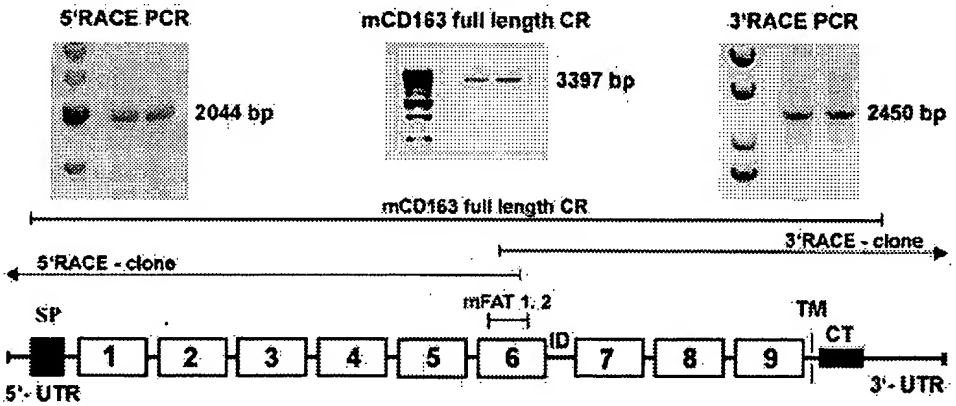

Fig. 1 Structure of the mouse *CD163* cDNA. The 5′- and 3′-RACE-derived cDNA clones used to establish the sequence are shown together with the amplified full-length coding region and a schematic representation of the domain structure of *mCD163*. The positions of the RACE cDNA clones are indicated as *solid dashes*. The respective PCR products (two independent reactions) are shown. The initial cDNA clone (mFAT 1.2) isolated by random PCR screening and the amplified full-length coding sequence are indicated as *solid lines* (*SP* signal peptide, *1–9* scavenger receptor cysteine-rich domains, *ID* scavenger interspersed domain, *TM* transmembrane domain, *CT* cytoplasmic tail, *3′/5′-UTR* untranslated regions)

mCD163 (Microsynth, Balgach, Switzerland). The resulting full-length coding cDNA was subsequently cloned by long-distance PCR using primers near both ends of the sequence of interest (forward primer: 5′-ATGGGTGGACACAGAATGGT; reverse primer: 5′-TTCCATTTAGCTGGCTGTCC). To determine the 5′ extent of the cDNA, six clones from three independent 5′-RACE reactions were isolated and sequenced from the 5′ end. After sequence alignment the longest 5′ extent was used to designate the start of transcription (+1).

The sequence of the *mCD163* cDNA is 4379 nucleotides long (Fig. 2). It contains a single long open reading frame encoding a 1121-amino acid sequence and a 934 nucleotide-long 3′ untranslated region containing four putative polyadenylation signal sequences, 12, 184, 192, and 391 bp upstream of the poly(A) tail. The predicted start of translation at the first ATG codon of the open reading frame is in position +76. In contrast to the human *CD163* where translation preferentially starts at the second ATG (Law et al. 1993) due to a very weak context of the first ATG and consequent leaky scanning of the ribosomal unit, the first ATG in the mouse sequence fulfills the basic requirements for efficient ribosomal binding and subsequent initiation of translation (Kozak 1996).

Computer-assisted analysis of the deduced amino acid sequence revealed a putative N-terminal signal peptide of 38 amino acids (SignalP-Website http://www.cbs.dtu.dk/services/SignalP-2.0/), one transmembrane-spanning region of 23 amino acids at position 1046–1068, followed by a KRRR basic stretch-starting 53 amino acid-long intracellular domain. The extracellular domain contains 16 putative N-linked glycosylation sites, 9 of which are conserved in the human sequence. The extracellular domain organization features nine SRCR domains, which are contiguous, except for a 31 amino acid-long scavenger interspersed domain (SID) containing one disulfide bond separating SRCR domains 6 and 7.

While different fold prediction models failed to recognize the CD163 SRCR domains, probably because they have not yet included the only suitable template structure currently available in the Protein Structure Database (PDB) in their template list, a FastA search of the Brookhaven PDB (at http://www.rcsb.org/) with any of the nine SRCR repeats in the mCD163 extracellular sequence revealed the X-ray structure of the M2BP SRCR domain (PDB entry 1BY2, 2 Å resolution; Hohenester et al. 1999) as a potential template for homology modeling. With sequence similarities between 37% (mCD163 SRCR8 to PDB1BY2) and 62% (mCD163 SRCR7 to PDB1BY2), reasonable models could be generated for all nine domains. The three-dimensional protein structures of the mCD163 SRCR domains were predicted by homology modeling using the Homology, Biopolymer and Discover modules of the program InsightII version 98 (Biosym/MSI, San Diego, Calif.). While the M2BP SRCR domain contains only three of the four disulfide bridges commonly found in group B SRCR domains, the coordinates of the residues corresponding to the remaining pair of cysteines were compatible with the remaining disulfide without major structural adjustments

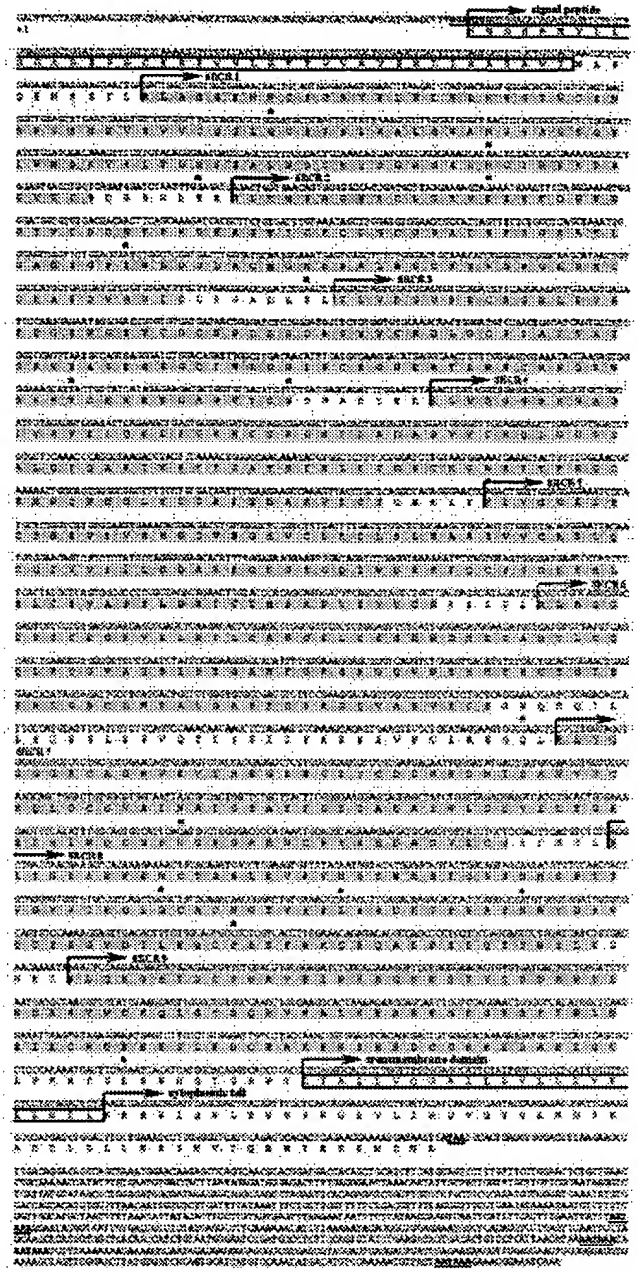

Fig. 2 Complete cDNA sequence, deduced amino acid sequence, and domain structure of mouse *CD163*. The Kozak consensus ribosomal-binding sequence is highlighted in *bold* and indicated as translation initiation site (ATG). The stop codon and four putative polyadenylation signals (AATAAA) are *underlined*. The predicted signal peptide, scavenger receptor cysteine-rich domains 1–9, and the transmembrane domain are marked. *Asterisks* indicate the 16 potential N-linked glycosylation sites

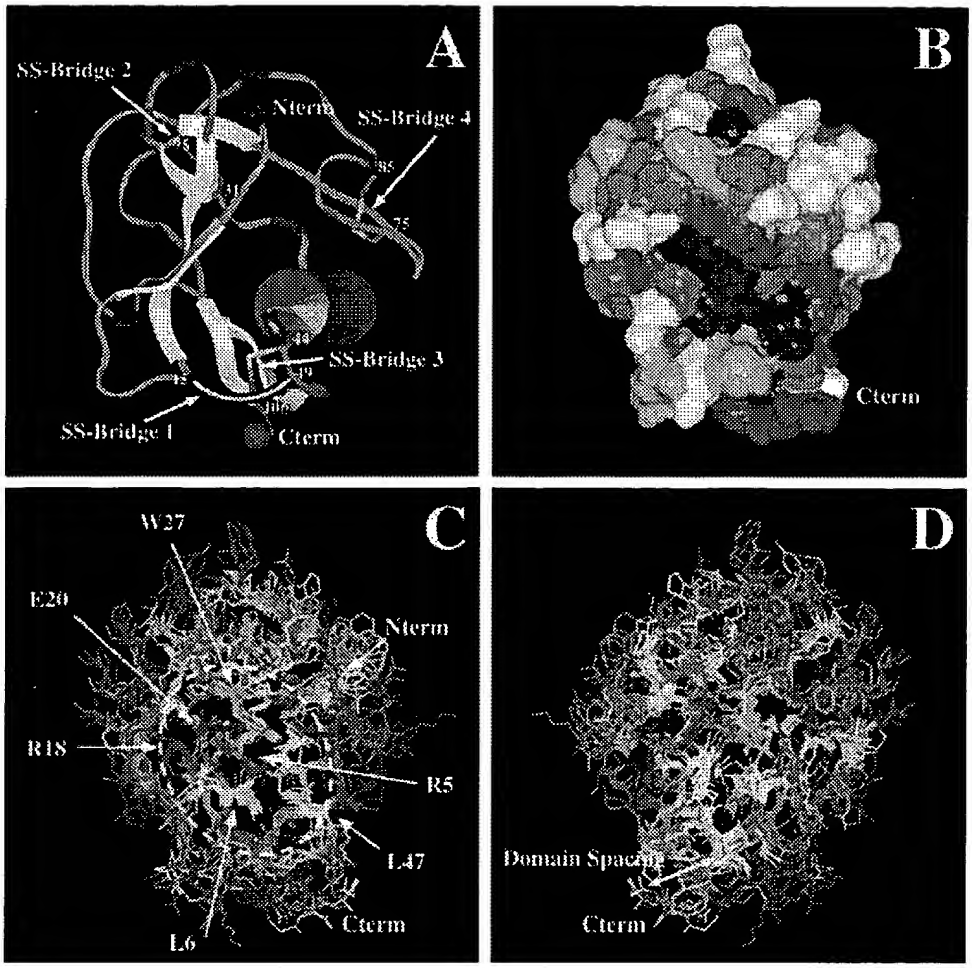

Fig. 3 **A** Cartoon representation of the M2BP SRCR domain structure (*red* α helix, *yellow* β sheets, *blue* turns, *green* random coil, *blue sphere* N-terminal end of the domain, *red sphere* C-terminal end). The residue numbers of the cysteine positions correspond to the generic numbering shown in Fig. 5A. The termini of the M2BP SRCR domain X-ray structure (PDB entry 1BY2, 2 Å resolution; Hohenester et al. 1999) have been truncated back to the domain boundaries suggested by the sequence alignment shown in Fig. 5. SS-bridge 1 is missing in the M2BP SRCR domain, as it is in other group A SRCR domains, although it is conserved in most group B SRCR domains. However, the position and orientation of the corresponding residues in the M2BP SRCR domain are compatible with a disulfide bond easily accommodated without any major structural changes. **B** Surface variability of the CD163 SRCR domains. The variability of individual amino acids (Fig. 5) is mapped onto a space-filling model of a representative mCD163 SRCR domain [*dark blue* identical in all nine domains of human and mouse CD163, *blue* strongly conserved, *green* somewhat conserved, *yellow* variable, *orange* very variable, *red* length variability (insertions/deletions)]. **C,D** Superposition of the nine mouse CD163 SRCR domain models. Two opposite views of all nine superimposed mCD163 SRCR domains are shown [*orange* aromatic residues (W, Y, F), *yellow* other hydrophobic residues (V, L, I, P, A, M, C), *green* uncharged hydrophilic residues (G, S, T, N, Q), *red* acidic residues (D, E), *blue* basic residues (H, K, R), *white* N- and C-terminal of the domains)]. While most of the domain surface shows the variability expected for the degree of sequence divergence between the different domains, the surface area indicated by the *dashed circle* (residues R5, L6, R18, E20, W27, and L47) is disproportionally conserved (**C**). The domains are closely spaced; maximally four residues are conformationally flexible in the area between the last Cys and the N-terminal of the next domain (indicated by the *double arrow* in **D**)

(Fig. 3A). Based on these models, all the disulfide pairings in the CD163 extracellular domain could be assigned. Some minor length variability had to be accommodated by loop remodeling, but all steric clashes introduced by side chain substitutions could be relieved by selecting appropriate side chain rotamers of the substituted or neighboring side chains. SRCR domain 4 of the mouse, but not the human CD163, lacks the first of the four disulfide bridges, since cysteine (Cys)381 has been replaced by a glycine residue, leaving Cys410 unpaired. SRCR domain 8 lacks disulfide bond 2 in both the mouse and the human protein.

To determine structurally and functionally important residues in the CD163 molecule we identified evolutionary conserved residues in the aligned sequences of all mouse and human CD163 SRCR domains (Fig. 4). This approach is based on two

observations: First, multiple homologue domains of a single protein derive from a common ancestor by gene duplication and subsequently undergo sequence divergence by random mutations. Second, as active-site residues are under evolutionary pressure to maintain their functional integrity they undergo distinctly fewer mutations than less functionally important amino acids, implying that evolutionarily related sequences can be compared with one another to extract structural and functional data (Lichtarge et al. 1996). The interdomain variability of each amino acid at a given position was calculated using the algorithm described by Wu and Kabat (1970) (variability=number of different amino acids at a given position/frequency of the most common amino acid at this position). After mapping of the calculated and color-coded sequence variability of each amino acid residue onto a space-filling SRCR domain model, a spatial cluster of highly conserved amino acids could be identified (Fig. 3B). The same conserved surface patch was identified by analysis of the superposition of the nine CD163 SRCR domain models and is indicated by the dashed circle in Fig. 3C. Given the progressive sequence divergence among homologue domains in the absence of an evolutionary pressure to maintain functional integrity, the observed degree of structural conservation implies an involvement of the described surface region in important intra- or intermolecular interactions. One may speculate that the identified surface patch is critically involved in ligand binding or in the three-dimensional arrangement of the nine SRCR domains. The conservation of core and structurally important residues corresponds to what one would expect for structurally homologous domains at the observed level of sequence divergence.

After confirmation of the strong inductive potential of dexamethasone on the expression of *mCD163* mRNA by RNAse protection assay (Fig. 5A), we used quantitative real-time RT-PCR to further determine the regulation of *mCD163* mRNA abundance by the anti-inflammatory mediators IL-4 and IL-10. Ribonuclease protection assays were performed with the Ambion Hybspeed kit according to the manufacturer's instructions, using a 300-bp *mCD163*-specific ^{32}P-labeled RNA probe (forward primer: 5′-TAATACG ACTCACTATAGGGAGGACCTGAGCAGATGAC AGAGG; reverse primer: 5′-ACCTGAGCAGATGA CAGAGG). For quantitative RT-PCR, equal amounts of total RNA from differently treated cells were reverse transcribed and amplified with gene-specific primers by real-time PCR using the LightCycler System (Roche) with SYBR Green I fluorescence (Roche) as described elsewhere (Staege et al. 2000) (*mCD163* forward primer: GGGAAGAGTGGAGCT CAAGA, reverse primer: ACCAGCTCCTTTCCCA AAAT; *hCD163* forward primer: 5′-ACATAGATC ATGCATCTGTCATTTG, reverse primer: 5′-CATTC TCCTTGGAATCTCACTTCTA). These experiments confirmed a more than tenfold induction of *mCD163* mRNA upon incubation with dexamethasone for 16 h compared to untreated cells. While IL-10 similarly induced *CD163* mRNA, the expression was only slightly suppressed below baseline levels by IL-4 after 16 h (Fig. 5B). The suppressive effect of IL-4 compared to control cells increased after prolonged culture of macrophages in the presence of IL-4, as the expression of *CD163* mRNA increases over time in untreated cells (data not shown). Further experiments revealed a parallel regulation of both mouse and human *CD163* genes (Fig. 5B, C).

The tissue distribution of *mCD163*-expressing cells was studied by in situ hybridization using a 1.5-kb *mCD163*-specific ^{35}S-CTP-labeled sense and anitsense RNA probe, as described elsewhere (Mueller et al. 1988). The highest level of constitutive *mCD163* expression was repeatedly observed in Kupffer cells of the liver. Furthermore, we identified a subset of red pulp macrophages and cells of the extrafollicular area of mesenteric lymph nodes that expressed *CD163*. Scattered *mCD163*-positive cells were located in the lamina propria of the colon and in the thymic cortex (data not shown). These results confirm the monocyte/macrophage-restricted expression pattern of *mCD163*.

Glucocorticoids induce a distinct subpopulation of alternatively activated macrophages expressing a specific set of molecules enabling them to actively participate in anti-inflammatory processes, immunosuppression, tolerance induction, and wound healing (Goerdt and Orfanos 1999). Despite the growing knowledge about the molecular repertoire of these suppressor macrophages involving the expression of various anti-inflammatory mediators and the lack of immunostimulatory effector molecules, the immunomodulating armature of these cells is still not fully understood.

The CD163 molecule can be assigned to a subgroup of group B SRCR members consisting almost entirely of a series of contiguous SRCR domains. Members of this subgroup are CD5, CD6, WC1, Spα, and several proteins encoded by the *DMBT1* gene locus. The most closely related proteins are WC1, Spα, and DMBT1. WC1 is involved in γδT-cell regulation (Kirkham et al. 1998; Wijngaard et al. 1992), CD5 and CD6 modulate T-cell activation after specific binding to their ligands CD70 and ALCAM (activated leukocyte cell adhesion molecule), respectively (Osorio et al. 1997; Whitney et al. 1995). Spα, a soluble protein consisting of three SRCR domains, is expressed by macrophages and suggested to inhibit thymocyte apoptosis upon various proapoptotic stimuli in mice (Gebe et al. 1997; Miyazaki et al. 1999). The *DMBT1* gene locus encodes the human DMBT1 and gp-340. While gp-340 is suggested to be a putative opsonin receptor for lung surfactant protein (Holmskov et al. 1999), DMBT1 has been proposed as a candidate tumor suppressor gene implicated in the pathogenesis of brain and various lung and gastrointestinal tumors. This conclusion is based on the finding of homozygous deletions and lack of expression in glioblastoma multiforme and

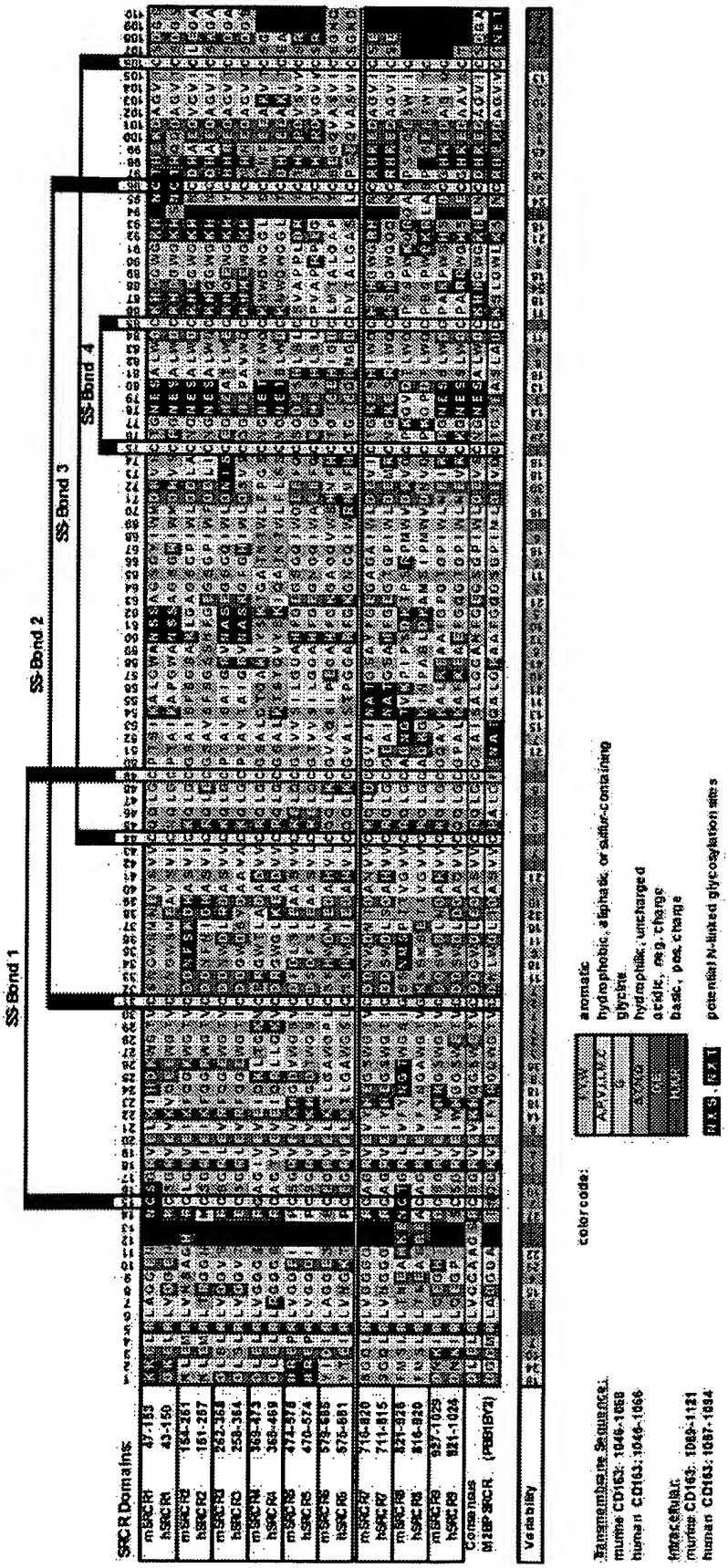

Fig. 4 CD163 SRCR sequence alignment and interdomain amino acid variability. Sequence alignment of the human and mouse CD163 scavenger receptor cysteine-rich domains and predicted disulfide bridges based on the homology to the M2BP SRCR domain structure (PDP entry 1BY2). Potential N-linked glycosylation sites are highlighted. Of the two potential sites of the M2BP SRCR domain, only the first is actually glycosylated. For each residue, the variability among all mouse and human CD163 was calculated using the Kabat algorithm and is indicated as 1 (identical in all nine domains of human and mouse CD163) to 54 (highest observed variability) (*dark blue* identical in all nine domains of human and mouse CD163, *blue* strongly conserved, *green* somewhat conserved, *yellow* variable, *orange* very variable)

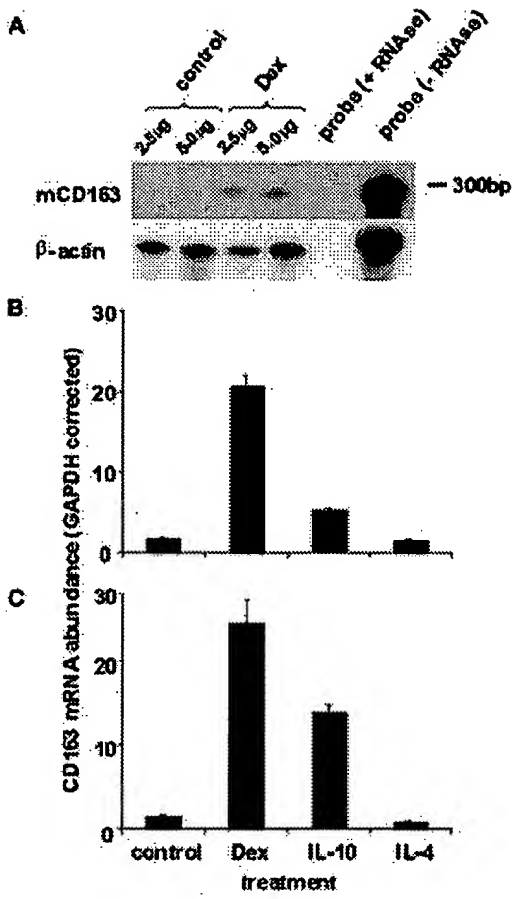

Fig. 5A–C Regulation of mouse and human *CD163* mRNA abundance by anti-inflammatory mediators. **A** RNAse protection assay of dexamethasone-treated and control mouse peritoneal macrophages: the 300-bp RNA probe consisting of the whole cDNA sequence of SRCR6 was incubated with 5.0 µg and 2.5 µg of total cellular RNA. As positive and negative controls the assay was performed without tester RNA, with and without RNAse treatment. **B,C** Real-time quantitative RT-PCR [mouse CD163 (**B**) human CD163 (**C**)]: relative glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *CD163* mRNA abundance were determined for each sample by inclusion of a serial dilution of cDNA in each PCR run as a standard curve. All values of *CD163* mRNA abundance are corrected for variations in *GAPDH* concentration. Indicated are the mean and SD of triplicate values from one representative experiment. Cells were incubated for 16 h with the indicated stimuli (dexamethasone 2.5×10^{-7} M, IL-4 10 ng/ml, IL-10 10 ng/ml)

medulloblastoma (Mollenhauer et al. 1997). Similar results were obtained in a significant fraction of gastrointestinal and lung tumors (Mori et al. 1999). Currently, data indicating putative functions of CD163 are very limited. In addition to the strong induction of *CD163* by glucocorticoids and the anti-inflammatory mediator IL-10, macrophages expressing high levels of CD163 have been identified in chronically inflamed tissues and during the wound-healing process (Zwadlo et al. 1987), indicating a possible role of this protein in the downregulation of the inflammatory process. Furthermore, exceptionally high levels of CD163 are expressed in human placental and alveolar macrophages. In the healthy organism, these alternatively activated macrophages function to protect the respective organ from detrimental inflammatory and immune reactions (Chang et al. 1993; Mues et al. 1989).

Our identification and characterization of the mouse *CD163* sets the basis for the development of transgenic and knockout mouse models. These powerful tools may soon unravel the biological significance of CD163 in the observed downregulation of the inflammatory response associated with the occurrence of high CD163-expressing macrophages.

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Hintz, K. A. (1); Sulahian, T. H. FASEB Journal, (April 20, 2000) Vol. 14, No. 6, pp. A1143.
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Sulahian, T. H. (1); Hoegger, P.; FASEB Journal, (April 20, 2000) Vol. 14, No. 6, pp. A1143.
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Development of an ELISA to measure soluble CD163 in biological fluids

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Abstract

CD163 is a monocyte/macrophage restricted transmembrane glycoprotein and a member of the scavenger receptor cysteine-rich (SRCR) family of proteins. SRCR proteins are typically associated with the immune system. The regulation of CD163 by cytokines and glucocorticoids suggests that it plays a role in inflammatory processes. While CD163 is expressed as a membrane-bound protein, it has been shown to be actively shed from the surface of monocytes in a protease-dependent fashion when cells are stimulated with a phorbol ester. To better elucidate the function and biological importance of CD163, we have developed a solid-phase sandwich enzyme linked immunosorbant assay (ELISA) for the detection of soluble CD163 in biological fluids. This assay has good repeatability both within and between runs (coefficients of variation (CVs) of 3.2% and 7.1% or better, respectively). While detection of CD163 was inhibited by ethylenediamine tetraacetic acid (EDTA), CD163 immunoreactivity was not altered by the addition of heparin or hemoglobin. This report details the development of this novel assay for soluble CD163 and provides the first evidence of CD163 immunoreactivity in normal plasma and serum samples.

Keywords: CD163; ELISA; Monocytes; Inflammation; Glucocorticoid

1. Introduction

CD163 (also called M130, RM3/1 and p155) is a member of the SRCR group B family of proteins. Members of this family contain highly conserved scavenger receptor domains and include the type I scavenger receptor, CD5, CD6 and WC1 (found on bovine γδ T cells). SRCR proteins are typically associated with immune function and are expressed on a number of different cells of the immune system (Resnick et al., 1994). CD163 expression is restricted to cells of monocyte lineage and increases as monocytes mature into macrophages (Pulford et al., 1998). To date, CD163 protein has been detected only in humans and the marmoset *Callithrix jacchus*, although a porcine homolog has been reported (Zwadlo-Klarwasser et al., 1992; Sánchez et al., 1999).

Abbreviations: Scavenger receptor cysteine-rich (SRCR); Enzyme linked immunosorbant assay (ELISA); Coefficient of variation (CV); Ethylenediamine tetraacetic acid (EDTA); Phorbol 12-myristate 13-acetate (PMA); Soluble CD163 (sCD163); Monoclonal antibody (mAb); Fetal bovine serum (FBS); Dexamethasone (DEX)

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Treatment of cultured monocytes with glucocorticoids, IL-10 or IL-6 dramatically increases CD163 expression. In addition, glucocorticoids have also been shown to upregulate expression in vivo (Morganelli and Guyre, 1988; Zwadlo-Klarwasser et al., 1990; Högger et al., 1998; Zwadlo-Klarwasser and Schmutzler, 1998; Buechler et al., 2000; Sulahian et al., 2000). In contrast, treatment with phorbol 12-myristate 13-acetate (PMA), IFN-γ or TNFα decreases surface CD163 expression on monocytes (Morganelli and Guyre, 1988; Högger et al., 1998; Buechler et al., 2000). However, combining glucocorticoid treatment with IFN-γ increases CD163 expression over that induced by glucocorticoids alone (Morganelli and Guyre, 1988). While the specific function of CD163 remains unclear, the regulation of its expression by these pro- and anti-inflammatory mediators suggests that it plays an important role in the activation and/or resolution of inflammatory processes.

CD163, like most members of the SRCR family, is expressed as a membrane bound protein. However, it is unique in that it appears to be the only SRCR protein that is actively shed from the cell surface. Droste et al. (1999) have recently demonstrated that treatment with PMA induces a protease dependent shedding, which can be inhibited by protein kinase C inhibitors. This finding led us to investigate the possibility that soluble CD163 (sCD163) is present in the plasma of normal individuals. To this end, we have developed an ELISA that utilizes two CD163-specific monoclonal antibodies (mAbs) (Mac 2-158 and RM3/1) to measure sCD163 immunoreactivity in cell culture supernatants, plasma and serum.

2. Materials and methods

2.1. In vitro CD163 shedding

Mononuclear cells were isolated from whole blood using Ficoll-Hypaque ($d = 1.077$ g) density gradient centrifugation (Böyum, 1968). The cells were cultured for 2 days at 5% CO_2 and 37°C in Hepes buffered RPMI 1640 (Hazelton Biologicals, Lenexa, KS)/20 μg/ml gentamicin (Elkins-Sinn, Cherry Hill, NJ)/10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) supplemented with 1 ng/ml *Escherichia coli* lipopolysaccharide (serotype 0111:B4, Sigma, St. Louis, MO), 0.5 ng/ml IL-1β (Peprotech, Rocky Hill, NJ) and 200 nM dexamethasone (DEX) (Steraloids, Wilton, NH). After culture, mononuclear cells were harvested, washed and exposed to 10^{-8} M PMA (Sigma) for 2 h at 37°C. The supernatants were collected and stored at −70°C until use.

2.2. Preparation of plasma and serum

For plasma production, whole peripheral blood was drawn from normal healthy donors into heparinized syringes and spun down at $500 \times g$ for 10 min. Plasma was drawn off and used immediately or frozen at −70°C until use. For serum production, whole peripheral blood was drawn from normal healthy donors. Blood was allowed to clot for 2 h at room temperature, then spun down at $500 \times g$ for 10 min. Serum was drawn off and used immediately or frozen at −70°C until use. Informed consent was obtained from all donors in accordance with the Dartmouth College Institutional Review Board requirements.

2.3. sCD163 ELISA

Nunc-immuno maxisorp 96-well plates (USA Scientific, Ocala, FL) were coated with 100 μl of 2 μg/ml Mac 2-158 (murine IgG1, prepared as previously described (Morganelli and Guyre, 1988)) overnight at 4°C. Plates were then washed four times with wash buffer (PBS/0.05% Tween 20). Wells were blocked with 200 μl blocking buffer (PBS/10% FBS) for 30 min at room temperature. Plates were again washed three times. Plasma, serum or cell culture supernatant diluted in blocking buffer was added to a level of 100 μl and incubated overnight at 4°C. For some experiments, EDTA (Gibco, Grand Island, NY), hemoglobin (Sigma) or heparin (American Pharmaceutical Partners, Los Angeles, CA) was added before being loaded into the ELISA plate. After incubation, plates were washed four times. CD163 was then detected by adding 100 μl of 0.5 μg/ml biotinylated RM3/1 (murine IgG1, Bachem, King of Prussia, PA) and incubating for 1 h at room temperature. Plates were washed four times and 100 μl of 1/1000 streptavidin alkaline-phos-

phatase (Caltag, Burlingame, CA) was added. After a 30-min room temperature incubation, plates were washed four times and 100 μl PNPP developing solution was added (1 mg/ml *p*-nitrophenyl phosphate (Sigma) dissolved in 0.05 M Na_2CO_3, 1 mM $MgCl_2$, pH 9.75). After sufficient color development, 100 μl of 1 N NaOH was added to stop the reaction. Plates were then read on an MRX microplate reader (Dynatech Laboratories, Burlington, MA) at 405 nm.

2.4. Statistical analysis

For PMA treated vs. control culture supernatants and plasma vs. serum comparisons (Sections 3.1 and 3.2), Student's paired *t*-test was used to test for a significant difference between the means. For the studies involving EDTA, heparin and hemoglobin (Section 3.3) one-way analysis of variance and the Dunnett multiple comparison test were performed to test for differences between the means. In all cases, differences were considered significant if $p < 0.05$. All statistical analyses were performed using Instat 2.03 for the Macintosh (GraphPad Software, San Diego, CA).

3. Results

3.1. Measurement of in vitro derived sCD163

As treatment of monocytes with PMA causes rapid shedding of CD163 (Droste et al., 1999), we investigated whether this shed CD163 could be detected by ELISA. Fig. 1 shows sCD163 immunoreactivity in titrations of supernatants from PMA treated monocyte cultures as detected with the sCD163 ELISA described in Materials and methods. The ELISA curve remained linear ($R^2 > 0.99$) up to a 50% supernatant concentration and sCD163 was detectable at supernatant concentrations as low as 3.1%. In addition, supernatants from PMA-treated cultures had significantly higher sCD163 immunoreactivity than control cultures ($p < 0.05$). Reproducibility for within and between runs was established using a 10% dilution of PMA-treated supernatants from three different mononuclear cell donors. The CVs ranged from 2.5% to 3.1% (within run, $n = 8$) and from 3.5% to 5.3% (between runs, $n = 5$).

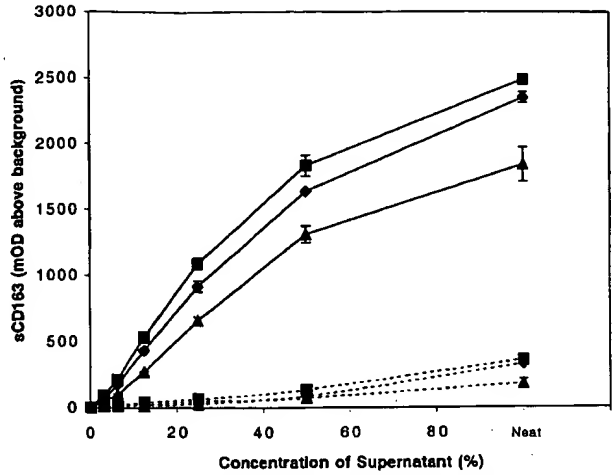

Fig. 1. sCD163 is present in the supernatant of PMA-treated mononuclear cells. Mononuclear cells from three donors were cultured as described in Materials and methods, then exposed to media with (solid lines) or without PMA (dotted lines). Each symbol represents sCD163 immunoreactivity levels (±the standard deviation) in a serial dilution of supernatant from a different mononuclear cell donor. The background signal has been subtracted.

Different mAb combinations were tested to compare the efficacy of Mac 2-158 and Mac 2-48 (both CD163-specific murine IgG1 mAbs) as capture antibodies (Fig. 2). As expected, capturing with Mac 2-48 and detecting with biotinylated Mac 2-48 gave no signal above background. The same was true for Mac 2-158. Furthermore, capturing with Mac 2-48 and detecting with biotinylated Mac 2-158 (or vise versa) also yielded no signal above background. sCD163 was detected only when either Mac 2-48 or Mac 2-158 was used to capture and biotinylated RM3/1 was used to detect. Of these two, Mac 2-158 captured more than 2.5-fold more sCD163 than Mac 2-48 at a 10% supernatant dilution and more than fourfold more at a 20% dilution (Fig. 3).

3.2. Measurement of sCD163 in serum and plasma

To determine if detectable levels of sCD163 are present in normal human plasma, samples from four different donors were titrated in blocking buffer. As seen in Fig. 4, sCD163 immunoreactivity was detectable at plasma concentrations as low as 2.5%,

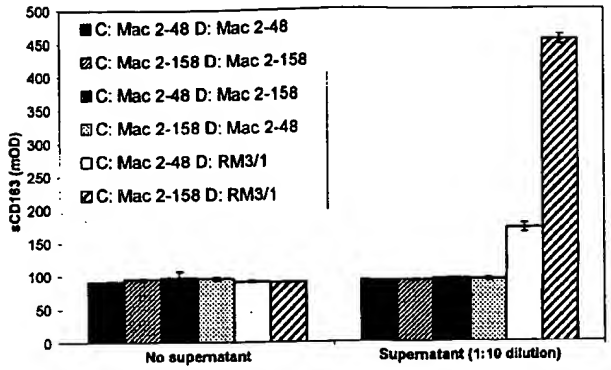

Fig. 2. Comparison of sCD163 ELISAs using different antibody combinations. Supernatants of PMA-treated mononuclear cells were diluted 10-fold and tested for sCD163 immunoreactivity by ELISA using different combinations of Mac 2-48, Mac 2-158 and RM3/1 to capture and detect sCD163. Data from one representative of three donors is shown. Bars represent sCD163 immunoreactivity ± the standard deviation of triplicate measurements.

while the ELISA was saturated at concentrations of plasma between 50% and 100%. In all samples, the sCD163 ELISA curve remained linear ($R^2 \geq 0.99$) at concentrations of plasma up to 17%, while the two samples with the lowest levels of sCD163 remained linear up to a 50% concentration. The CVs for within

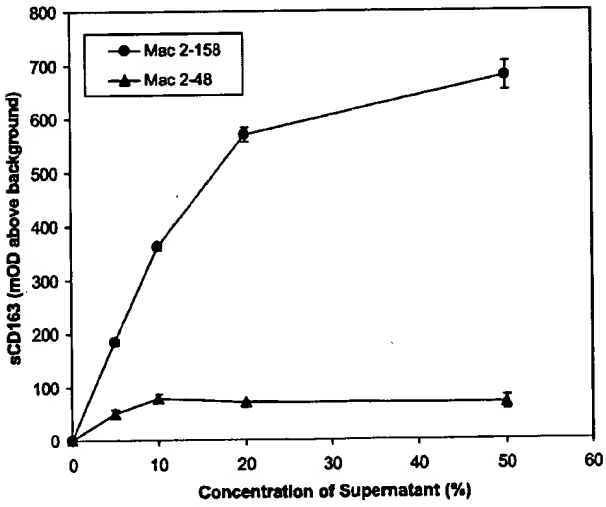

Fig. 3. Comparison of sCD163 ELISAs using Mac 2-158 or Mac 2-48 to capture. Titrations of supernatants of PMA-treated mononuclear cells were measured for sCD163 immunoreactivity using either Mac 2-48 or Mac 2-158 to capture and biotinylated RM3/1 to detect. Data from one representative of three donors is shown. Points represent sCD163 immunoreactivity ± the standard deviation of triplicate measurements with the background signal subtracted.

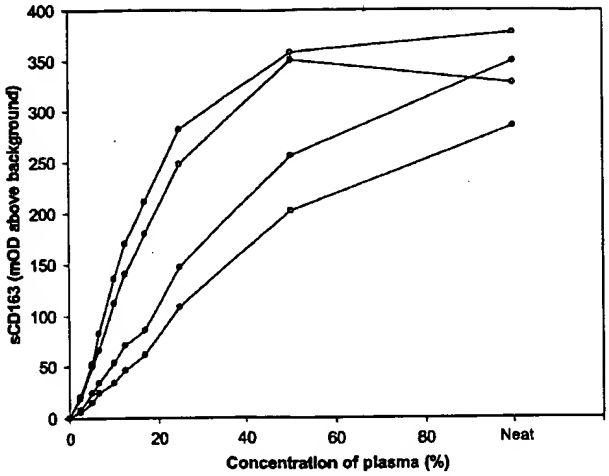

Fig. 4. sCD163 is present in normal human plasma. Plasma taken from four donors was titrated in blocking buffer and tested for sCD163 immunoreactivity by ELISA. Each line represents the sCD163 levels in a serial dilution of plasma from a different donor with the background signal subtracted.

and between runs at a 10% dilution of normal plasma were established for three different donors and ranged from 1.7% to 2.0% (within run, $n = 10$) and from 5.7% to 7.1% (between runs, $n = 5$). In addition, Fig. 5 demonstrates the specificity of the sCD163 ELISA when performed on normal plasma. Plasma samples from four donors were pre-incubated

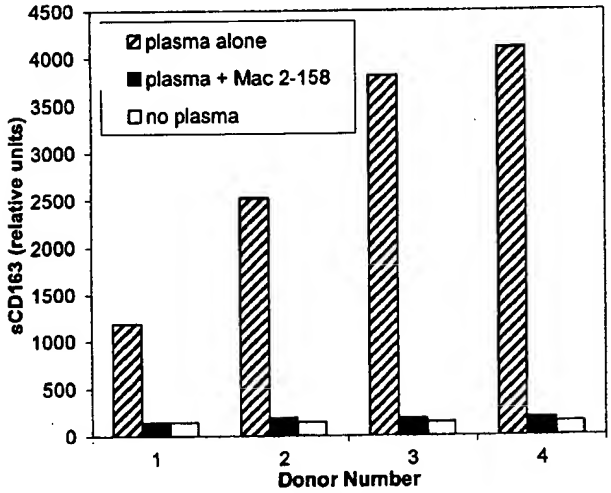

Fig. 5. Soluble Mac 2-158 blocks the detection of sCD163. Plasma samples from four donors were pre-incubated overnight with or without 25 μg/ml mAb Mac 2-158 and diluted 10-fold in blocking buffer before being tested for sCD163 immunoreactivity by ELISA.

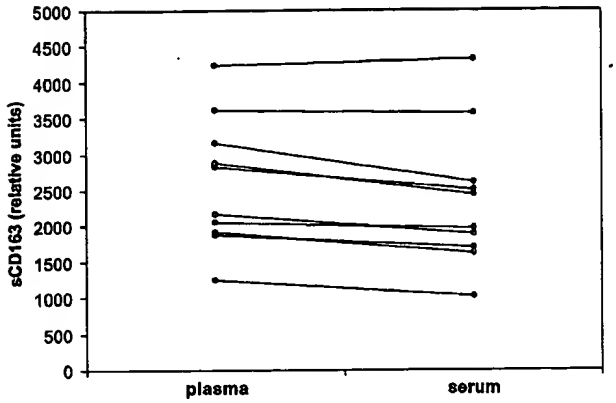

Fig. 6. Comparison of sCD163 detected by ELISA in plasma and serum. Plasma and serum were prepared from whole blood drawn from 10 donors. Samples were diluted 10-fold in blocking buffer and sCD163 levels were measured by ELISA. Each line represents the plasma and serum sCD163 immunoreactivity levels for a different donor.

overnight with or without 25 μg/ml mAb Mac 2-158 before being diluted with blocking buffer and analyzed by ELISA. In all cases, pre-incubation with Mac 2-158 reduced sCD163 immunoreactivity to background levels.

Because the measurement of blood proteins by ELISA can sometimes be affected either by anticoagulants found in plasma or by the clotting process, we tested paired blood samples drawn either in the presence or absence of heparin. The resulting serum and plasma were then analyzed for sCD163. Although the paired plasma and serum samples had a statistically significant difference in sCD163 immunoreactivity ($p < 0.01$), this difference was subtle (less than 10% of the total value) (Fig. 6). The CVs for within and between runs at a 10% dilution of normal serum were established for three different donors and ranged from 2.1% to 3.2% (within run, $n = 10$) and from 4.2% to 5.8% (between runs, $n = 5$).

3.3. Effect of EDTA, hemoglobin and heparin on the sCD163 ELISA

EDTA and heparin are common anticoagulants used to prepare clinical samples while hemoglobin is a common contaminant in plasma and serum preparations due to hemolysis. To address the possibility that these substances could interfere with the sCD163 ELISA, serum samples and supernatants from PMA-treated mononuclear cells were tested in the presence and absence of EDTA, hemoglobin or heparin. These substances were added to final concentrations of 1.25 mg/ml, 2.5 mg/ml and 5 USP units/ml, respectively. The samples were then analyzed by ELISA. Treatment with either hemoglobin or heparin did not significantly alter the sCD163 immunoreactivity of either the culture supernatants or serum samples (Fig. 7A and B). In contrast, EDTA caused a 13% ($p < 0.05$) and 28% ($p < 0.05$) decrease in the mean sCD163 immunoreactivity of the culture super-

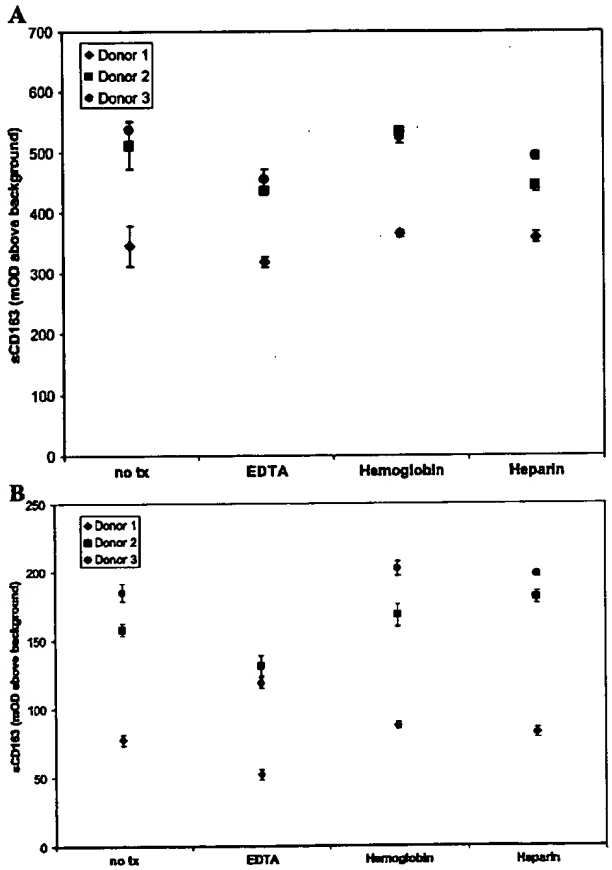

Fig. 7. Effect of EDTA, hemoglobin and heparin on sCD163 ELISA. Either (A) supernatants from PMA-treated mononuclear cell cultures or (B) serum samples were diluted 10-fold with blocking buffer before being spiked with EDTA (1.25 mg/ml), hemoglobin (2.5 mg/ml) or heparin (5 USP units/ml). sCD163 immunoreactivity was then measured by ELISA. Each point represents sCD163 immunoreactivity ± the standard deviation of triplicate measurements for a different mononuclear cell or serum donor with the background signal subtracted.

natants and serum samples, respectively. When higher concentrations (up to 5 mg/ml) of EDTA were used, a dose-dependent decrease in sCD163 immunoreactivity was detected in both culture supernatants and serum samples (data not shown).

4. Discussion

These studies confirm those of Droste et al. (1999), which demonstrated that sCD163 is detectable in the supernatant of PMA-treated monocytes. Also consistent is the finding that sCD163 immunoreactivity, as measured by ELISA, is higher in supernatants from PMA-treated mononuclear cells than from non-treated cultures. We have now extended those previous studies by providing the first evidence that significant amounts of sCD163 are present in normal human plasma and serum.

Of particular interest is the finding that, while both Mac 2-48 and Mac 2-158 are specific for CD163, an ELISA using these mAbs to capture and detect sCD163 failed to produce signals above background. This suggests that Mac 2-48 and Mac 2-158 bind to either identical or overlapping epitopes on the CD163 molecule. This is supported by flow cytometric studies, which show that Mac 2-48 and Mac 2-158 inhibit each other with respect to monocyte surface staining (data not shown). Since a sCD163 ELISA using either purified Mac 2-48 or Mac 2-158 and biotinylated RM3/1 is able to detect sCD163, it is likely that RM3/1 binds to a CD163 epitope distinct from that bound by Mac 2-48 and Mac 2-158. In addition, the finding that EDTA inhibits this ELISA suggests a role for calcium in the binding of one or more of these mAbs to sCD163, or that calcium is necessary for maintaining the proper tertiary structure of CD163. Regardless, it is clear that care must be taken when choosing an anticoagulant for clinical samples.

CD163 has been detected in both membrane-bound and secreted forms, and it is presently unknown whether its major biological functions are carried out as a secreted protein or as a cell surface receptor. In addition, it is possible that the shed molecule serves as an inhibitor of the membrane-bound form, similar to the soluble TNF-α receptor (Seckinger et al., 1990). As the shed form of CD163 may be of critical importance to the understanding of CD163, the ELISA described in the studies presented here may prove beneficial in elucidating the biology of this enigmatic molecule.

Acknowledgements

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